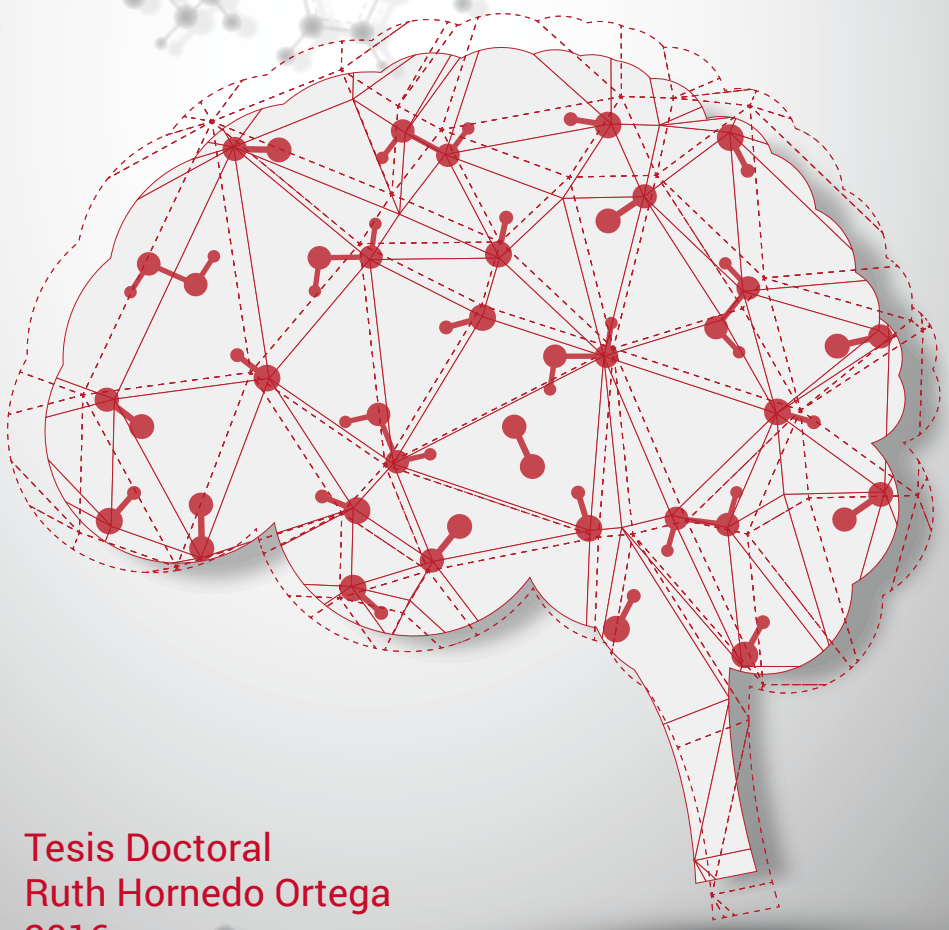
Caracterización Química, Propiedades Antioxidantes y Evaluación de los Efectos Neuroprotectores de Bebidas Fermentadas



Tesis Doctoral
Ruth Hornedo Ortega
2016

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Camila Renè Maggi
Graziella Tonucci

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UNIVERSIDAD DE SEVILLA
Facultad de Farmacia
Departamento de Nutrición y
Bromatología, Toxicología y
Medicina Legal

"Caracterización Química, Propiedades Antioxidantes y Evaluación de los Efectos Neuroprotectores de Bebidas Fermentadas"

Memoria que presenta
la Licenciada RUTH HORNEDO ORTEGA
para optar al Título de Doctor por la Universidad de Sevilla
con la Mención de "Doctor Internacional"

Sevilla, Julio 2016

Departamento de Nutrición y Bromatología, Toxicología y Medicina Legal

Dña. ANA Mª CAMEÁN FERNÁNDEZ, Catedrática de Universidad y Directora del Departamento de Nutrición y Bromatología, Toxicología y Medicina Legal de la Facultad de Farmacia de la Universidad de Sevilla.

INFORMA:

Que la Tesis Doctoral titulada "Caracterización química, propiedades antioxidantes y evaluación de los efectos neuroprotectores de bebidas fermentadas", presentada por la Lda. RUTH HORNEDO ORTEGA para optar al grado de Doctor por la Universidad de Sevilla con la Mención de "Doctor Internacional", ha sido realizada en el Área de Nutrición y Bromatología de este Departamento bajo la dirección de las Dras. Mª CARMEN GARCÍA PARRILLA Y ANA Mª TRONCOSO GONZÁLEZ, cumpliendo los requisitos exigidos.

Y para que conste, firmo el presente en Sevilla,
Julio de 2016.

Fdo. Ana Mª Cameán Fernández

Departamento de Nutrición y Bromatología, Toxicología y Medicina Legal

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INFORMAN:

Que la Tesis Doctoral titulada "Caracterización química, propiedades antioxidantes y evaluación de los efectos neuroprotectores de bebidas fermentadas", ha sido realizada por la Lda. RUTH HORNEDO ORTEGA en el Departamento de Nutrición y Bromatología, Toxicología y Medicina Legal de la Universidad de Sevilla, bajo su dirección y que reúne, a su juicio, las condiciones requeridas para optar al grado de Doctor por la Universidad de Sevilla con la Mención de "Doctor Internacional".

Y para que conste, firman el presente en Sevilla,
Julio de 2016.

Fdo. Mª Carmen García Parrilla

Fdo. Ana Mª Troncoso González

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A mi Abuelita

"Ama muchas cosas, porque en amar existe la verdadera fuerza y quien ama mucho logrará mucho, y lo que se hace con amor, está bien hecho"

Vincent van Gogh

Índice

Resumen

La fresa es una excelente fuente de compuestos bioactivos, sin embargo, es un fruto muy perecedero. La elaboración de productos fermentados supone una valiosa estrategia para disminuir las pérdidas económicas por excedentes de fruta y aumentar su vida útil.
Por otro lado, resulta de gran interés el desarrollo de procesos biotecnológicos que preserven los compuestos bioactivos presentes en el fruto de partida.

En esta Tesis Doctoral se ha evaluado la composición antociánica, color y actividad antioxidante de productos fermentados elaborados a partir de puré de fresa, concretamente, fermentados alcohólicos, acéticos y glucónicos, así como la estabilidad de una bebida obtenida a partir de estos productos.
Asimismo, se ha estudiado el efecto neuroprotector de estos fermentados frente a la toxicidad del péptido β-amiloide (Aβ) y a la producción de Especies Reactivas de Oxígeno (ROS).
Por otro lado, se ha demostrado la bioactividad del ácido protocatéquico (uno de los principales metabolitos de antocianos) frente a la agregación y toxicidad del péptido Aβ y de la proteína α-sinucleína (αS) *in vitro*.

Además, se ha analizado el efecto de otros compuestos bioactivos (melatonina y otros compuestos indólicos) presentes en fresa y otros productos fermentados frente al proceso de fibrilación y neurotoxicidad del péptido Aβ.

Los resultados obtenidos han permitido la identificación de nuevos compuestos antociánicos en fermentados alcohólicos y acéticos. Al mismo tiempo, se ha comprobado cómo el proceso que mejor preserva los antocianos es la fermentación glucónica, que se ha seleccionado como base de la bebida elaborada.
Además, los fermentados alcohólicos y glucónicos protegen a las células neuronales de la toxicidad del péptido Aβ. Asimismo, el ácido protocatéquico posee un efecto inhibidor de la formación de fibras de Aβ y αS y efectos neuroprotectores demostrado por diversas técnicas *in vitro.* Por último, el estudio de los compuestos indólicos frente al proceso de agregación y la toxicidad del Aβ ha puesto de manifiesto que la serotonina es un potente inhibidor de la formación de fibras y que la melatonina y los compuestos indólicos relacionados con su síntesis presentan un efecto preventivo frente a la muerte neuronal producida por este péptido.

Summary

The strawberry is an excellent source of bioactive compounds, however, it is a very perishable fruit. The elaboration of fermented products supposes a valuable strategy to prevent the economic losses due to strawberry surplus and to increase the shelf life.
On the other hand, the use of biotechnological processes that preserve the bioactive compounds is a relevant aspect to take into consideration.

In this PhD Thesis, anthocyanin composition, colour and antioxidant activity of different fermented products made from strawberry purée by alcoholic, acetic and gluconic fermentations have been evaluated. Additionally, the stability of a beverage obtained from these products has been studied. Moreover, the neuroprotective effect of these products against amyloid-β (Aβ) toxicity and Reactive Oxygen Species (ROS) production has been investigated.
In addition, the bioactivity of protocatechuic acid (one of the most important anthocyanin metabolite) against the aggregation and toxicity of Aβ peptide and α-synuclein (αS) protein *in vitro* has been demonstrated.

Furthermore, it has been confirmed that other bioactive compounds (melatonin and indolic related compounds) described in strawberry and in other fermented products present an effect on the inhibition of the Aβ aggregation and the Aβ-induced toxicity.

The obtained results have led to the identification of new anthocyanin compounds in alcoholic and acetic fermented products. Moreover, it has been proven that gluconic fermentation is the process, from those studied in this Thesis, that better preserves the anthocyanin profile.
Thus, it was the one selected as substrate to elaborate the beverage. Furthermore, alcoholic and gluconic fermented extracts protect neuronal cells against Aβ toxicity. Protocatechuic acid possesses an inhibitory effect of the Aβ and αS fibril formation and neuroprotective properties demonstrated by several *in vitro* techniques. Finally, serotonin has been proved to be a potent inhibitor of the fibril formation. Melatonin and the related indolic compounds presented a preventive effect against the cell death produced by Aβ.

Abreviaturas

6-ODDA: 6-hidroxidopamina

ACh: Acetilcolina

AD: Enfermedad de Alzheimer (Alzheimer Disease)

Ala30Pro: Alanina 30 Prolina

Ala42: Alanina 42

Ala53Thr: Alanina 53 Treonina

APOE: Alipoproteína E

APP: Proteína Precursora de Amiloide (Amyloid Protein Precursor)

Arg1441Cys: Arginina 1441 Cisteína

Arg1441Gly: Arginina 1441 Glicina

Asp672: Aspargina 672

AT: Antocianos Totales

Aβ: péptido β-amiloide

$A\beta_{1-28}$:péptido β-amiloide fragmento 1-28

$A\beta_{25-35}$: péptido β-amiloide fragmento 25-35

$A\beta_{25-40}$: péptido β-amiloide fragmento 25-40

$A\beta_{40:}$:péptido β-amiloide fragmento 1-40

$A\beta_{42}$: péptido β-amiloide fragmento 1-42

BEDCA: Base de Datos Española de Composición de Alimentos

BHE: Barrera Hematoencefálica

Ch: Colina

ChAT: Acetilconiltransferasa

CL: Cuerpos de Lewy

C_{max}: Concentración máxima

EC: Comisión Europea

EGCG: Epigalocatequina 3-galato

Glu46Lys: Glutamina 46 Lisina

GLUT2: transportador de glucosa 2 (Glucose Transporter 2)

Gly2019Ser: Glicina 2019 Serina

GSH: Glutatión

H_2O_d: agua destilada

ID: Intestino Delgado

IDR: Ingesta Diaria Recomendada

Ile2020Thr: Isoleucina 2020 Treonina

Ile41: Isoleucina 41

IPF: Índice de Polifenoles Totales

LRRK2: Leucine-rich repeat kinase 2

Met671: Metionina 671

MPDP+: 1-metil-4-fenil-2,3-dihidropiridinio

MPP+: 1-metil-4-fenilpiridinio

MPTP: 1-metil-4-fenil-1,2,3,6 tetrahidropiridina

NAC: Componente no amiloidogénico (Non Amyloid Component)

PD: Enfermedad de Parkinson (Parkinson Disease)

PSEN1: Presenilina 1

PSEN2: Presenilina 2

ROS: Especies Reactivas de Oxígeno (Reactive Oxygen Species)

SGLT1: Sodium-Glucose Linked Transporter 1

SN: Sistema Nervioso

SNC: Sistema Nervioso Central

SNc: Sustancia Negra compacta

SNCA: gen α-sinucleína

TEM: Micorscopía Electrónica de Transmisión (Transmission Electronic Microscopy)

ThT: Tioflavina T

TPK: Tau-protein kinase

Tyr1699Cys: Tirosina 1699 Cisteína

U.S.: United States

UE: Unión Europea

UMA: Unidades de Masa Atómica

Val40: Valina 40

αS: proteína α-sinucleína

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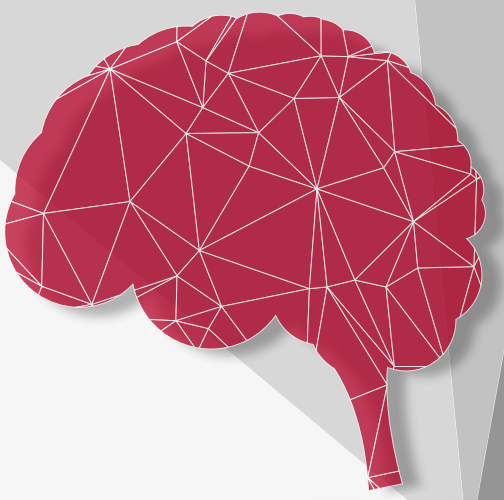
Introducción



1. Fresa

La fresa (*Fragaria x ananassa*) es una planta estolonífera de la familia *Rosaceae* de tipo herbáceo y perenne. Su fruto, de forma triangular, es un agregado formado por la maduración conjunta de varios ovarios, que pertenecen a una única flor y adheridos a un receptáculo pulposo o hipantio que soporta los ovarios. Inicialmente el fruto es blanco con tonos verdosos y con la maduración evoluciona hacia el rojo (Mazza & Miniati, 1993).

A nivel mundial, la producción de fresa es de aproximadamente 2.5 millones de toneladas siendo los mayores productores: Estados Unidos, España, Polonia, Japón, Italia, Rusia y Corea. Según los datos de producción publicados por el Ministerio de Agricultura y Medio Ambiente del Gobierno de España (2015), la producción de fresa y fresón alcanzó las 312.466 toneladas durante el año 2014. En Andalucía y, concretamente en Huelva, se concentra el 90 % de la producción nacional.

1.1. Composición nutricional de la fresa

La fresa es considerada una excelente fuente de nutrientes, compuestos bioactivos y fibra.

Su composición nutritiva se resume en la Tabla 1. La fresa es un fruto poco energético, ya que está compuesta mayoritariamente por agua. Los hidratos de carbono (azúcares y fibra) suponen el 80 % de su composición en peso seco. Además es muy rica en vitamina C, pudiendo superar las Ingestas Diarias Recomendadas (IDR) para esta vitamina (60 mg) con el consumo de una ración de fresa que equivale a 150 g. También contiene cantidades importantes de ácido fólico (vitamina B_9).

Entre los minerales, se puede destacar que posee elevadas cantidades de potasio, fósforo, calcio, magnesio y bajas cantidades de sodio.

Nutrientes	Valor/100 g de porción comestible
Energía (Kcal)	36
Grasa total (lípidos totales) (g)	0,5
Proteínas (g)	0,7
Agua (g)	89,6
Hidratos de carbono (g)	7,68
Fibra, dietética total (g)	2,0
Azúcares, de los cuales (g):	4,89
Sacarosa (g)	0,47
Glucosa (g)	1,99
Fructosa (g)	2,77
Grasas	
Ácido grasos, monoinsaturados totales (g)	0,063
Ácido grasos, poliinsaturados totales (g)	0,24
Ácido grasos, saturados totales (g)	0,032
Vitaminas	
Vitamina A, equivalentes de retinol y carotenoides (µg)	1
Vitamina D (µg)	0
Vitamina E, equivalentes de alfa tocoferol (mg)	2
Folato total (µg)	20
Equivalentes de niacina, totales (mg)	0,6
Rivoflavina (mg)	0,04
Tiamina (mg)	0,02
Vitamina B_{12} (µg)	0
Vitamina B_6 (mg)	0,06
Vitamina C (ácido ascórbico) (mg)	60
Minerales	
Calcio (mg)	25
Hierro (mg)	0,8
Potasio (mg)	190
Magnesio (mg)	12
Sodio (mg)	2
Fósforo (mg)	26
Ioduro (µg)	8
Selenio, total (µg)	Trazas
Zinc (mg)	0,1

Tabla 1.

Composición nutritiva de la fresa. Valores correspondientes a la variedad *Fragaria Vesca* por 100 g de porción comestible. Fuente: Base de Datos Española de Composición de Alimentos (BEDCA) y Datos de la United States (U.S.) Department of Agriculture, Agriculture Research Service.

1. 2. Compuestos Polifenólicos de la fresa

Además de su composición nutritiva, la fresa es una abundante fuente de compuestos polifenólicos. Estos compuestos son metabolitos secundarios y se encuentran en hojas, frutos, semillas, tallos, etc. Entre sus funciones se encuentran la actuación como agentes de defensa ante estímulos muy diversos como el estrés y la luz y son los responsables del color.

Los compuestos polifenólicos son un grupo heterogéneo de sustancias. Han sido descritos unos 8000 distintos y varían ampliamente en su estructura química la cual está caracterizada por la presencia de uno o varios anillos fenólicos.

Los tipos de polifenoles se clasifican en función del número de anillos fenólicos que poseen así como la localización de los sustituyentes y las correspondientes esterificaciones en su caso con azúcares y/o ácidos orgánicos (Strack & Wray, 1989).

Los compuestos fenólicos se clasifican en dos grandes grupos: No flavonoides y Flavonoides que a su vez se dividen en distintos subgrupos (Figura 1).

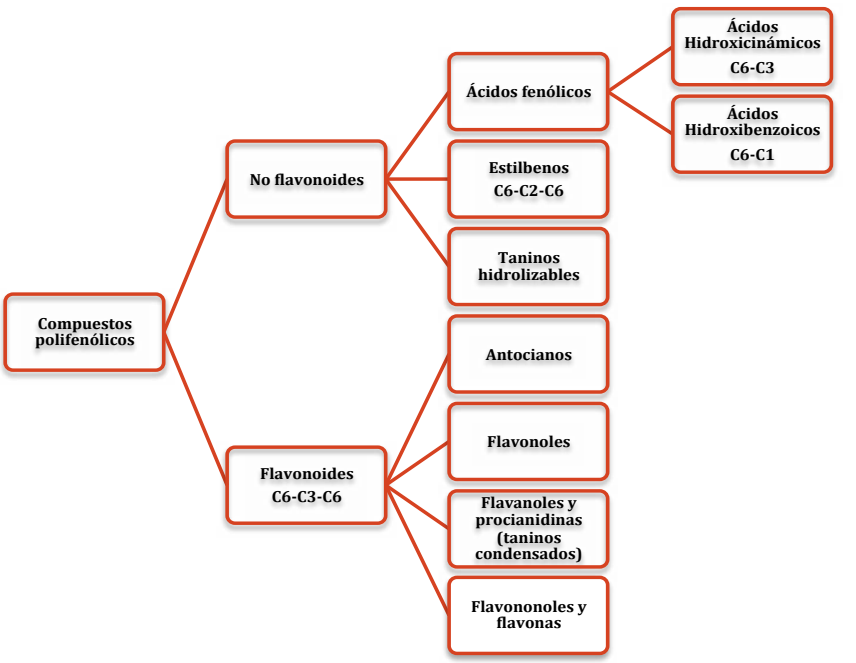


Figura 1: Clasificación de los compuestos polifenólicos.

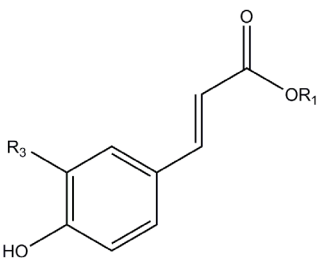
En general, la mayor parte de los compuestos polifenólicos de la fresa pertenecen al grupo de los flavonoides (mayoritariamente antocianos y flavonoles y flavanoles en menor medida), seguido por los taninos hidrolizables (elagitaninos y galotaninos) y el ácido elágico. También se encuentran en menor cantidad ácidos fenólicos (hidroxibenzoicos e hidroxicinámicos) y taninos condensados (Kähkönen et al., 2001; Määttä-Riihinen et al., 2004; Aaby et al., 2005).

1.2.1. Compuestos fenólicos no flavonoides

Los polifenoles no flavonoides incluyen moléculas sencillas como los ácidos fenólicos con un anillo fenólico ligado a esqueleto de entre 1 a 3 carbonos, así como otros compuestos formados por dos anillos fenólicos unidos por un puente de 2 carbonos como es el caso de los estilbenos. Por otro lado, se encuadran dentro de esta clasificación los taninos hidrolizables como los elagitaninos y galotaninos, siendo las estructuras más complejas de los compuestos no flavonoides.

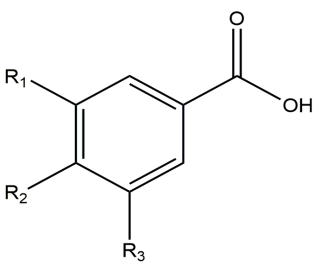
1.2.1.1. Ácidos fenólicos

Se han identificado en fresa, ácidos fenólicos derivados del ácido benzoico (ácidos hidroxibenzoicos) (C6-C1) y derivados del ácido cinámico (ácidos hidroxicinámicos) (C6-C3).
Entre los ácidos hidroxicinámicos destacan el ácido *p*-cumárico, ácido cinámico, ácido cafeico y ácido ferúlico y sus derivados (Mattila & Kumpulainen, 2002; Wang et al., 2002; Määttä-Riihinen et al., 2004; Ehala et al., 2005).
En el caso de los ácidos hidroxibenzoicos, el ácido gálico y sus derivados son los mayoritarios. El ácido elágico (dímero de ácido gálico) es un ácido hidroxibenzoico particularmente importante en la fresa, en la cual se puede encontrar tanto en forma libre como, más comúnmente, formando parte de elagitaninos (Häkkinen et al., 2000; Häkkinen & Törrönen, 2000; Törrönen, 2000; Williner et al., 2003; Kosar et al., 2004; Álvarez-Fernández et al., 2014)



Ácidos hidroxicinámicos	R_1	R_3
Ácido *p*-cumárico	H	H
Ácido cafeico	H	OH
Ácido ferúlico	H	OCH_3
Ácido cinámico	H	

Figura 2: Estructura química de los ácidos hidroxicinámicos.



Ácidos hidrobenzoicos	R_1	R_2	R_3
Ácido gálico	OH	OH	OH

Figura 3: Estructura química de los ácidos hidroxibenzoicos.

1.2.1.2. Estilbenos

Son compuestos con 14 carbonos en su estructura base y un esqueleto C6-C2-C6. Se han encontrado en fresa pequeñas cantidades de *trans*-resveratrol y del glucósido de *trans*-resveratrol (Ehala et al., 2005; Álvarez-Fernández et al., 2014).

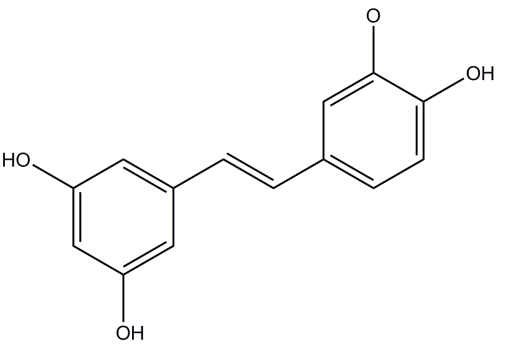


Figura 4: Estructura química del *trans*-resveratrol.

1.2.1.3. Taninos hidrolizables

Los taninos hidrolizables son polímeros de ácidos fenólicos esterificados con ácidos fenólicos (ácido gálico) y unidos a azúcares (glucosa generalmente) o a un polialcohol. Estos compuestos presentan una enorme variabilidad estructural con pesos moleculares de entre 500 y 3000 UMA. Dentro de este grupo destacamos por su presencia en fresa los elagitaninos. Los elagitaninos están formados por diferentes combinaciones del ácido gálico y del ácido hexahidroxidifénico (HHDP) con glucosa. Podemos encontrar desde estructuras más simples como el glucósido de ácido elágico hasta estructuras más complejas como es el caso del sanguiin H-6, siendo este último el más característico en fresa (Buendía et al., 2010).

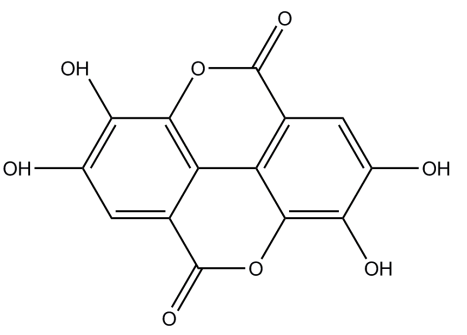


Figura 5: Estructura química del ácido elágico.

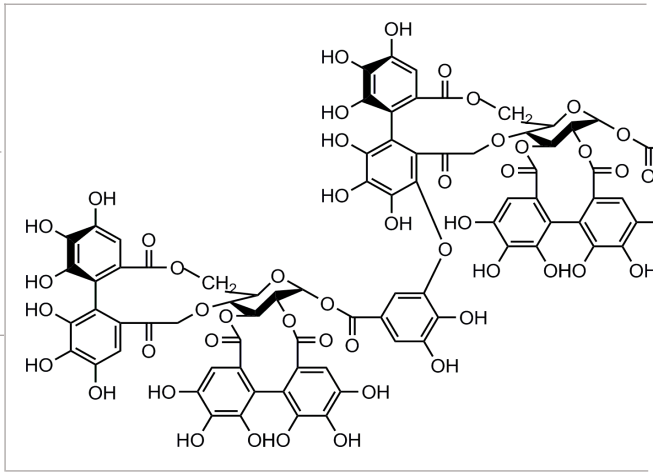


Figura 6: Estructura química del sanguiin H-6.

1.2.2. Compuestos fenólicos flavonoides

Son compuestos de bajo peso molecular con un esqueleto C6-C3-C6, compuesto por tres anillos: A y B, anillos fenilo y C, anillo heterocíclico pirano.

Los flavonoides se encuentran mayoritariamente como glicósidos aunque también pueden aparecer en su forma libre denominadas agliconas. Existen varios tipos de flavonoides, clasificados en base al estado de oxidación del anillo heterocíclico pirano y la posición del anillo B, así como por el número y posición de los grupos funcionales presentes y la unión a otras moléculas como azúcares, ácidos orgánicos, etc.

Los principales grupos son flavonoides son: flavonoles, flavanoles, flavononoles, flavonas y antocianidinas.

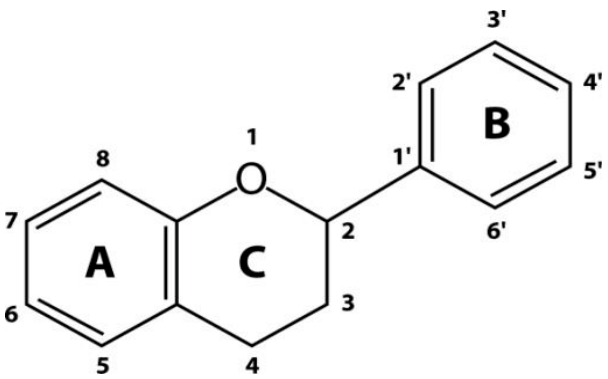
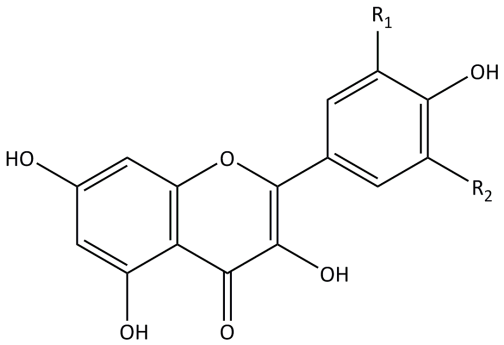


Figura 7: Estructura química general de los compuestos fenólicos flavonoides.

1.2.2.1. Flavonoles

Los flavonoles presentes en la fresa son mayoritariamente derivados de la quercetina y del kaempferol. Normalmente aparecen en forma glucosilada como es el caso del kaempferol 3-glucósido o la quercetina 3-glucósido (Wang et al., 2002; Määttä-Riihinen et al., 2004; Ehala et al., 2005). Se han identificado otros derivados de la quercetina en fresa como la quercetina 3-glucurónido y la quercetina rutinósido (Aaby et al., 2007; Del Bubba et al., 2012; Álvarez-Fernández et al., 2014).



Flavonoles	R_1	R_2
Quercetina	OH	OH
Kaempferol	H	H

Figura 8: Estructura química de los flavonoles.

1.2.2.2. Flavanoles (Flavan-3-ol) y procianidinas

Los flavanoles, pueden encontrarse en forma monomérica, dimérica (procianidinas) o polimérica (taninos condensados).
Dentro de estos primeros se han identificado en fresa cantidades importantes de (+)-catequina, (+)-galocatequina, (-)-epicatequina, (-)-epicatequina 3-galato y (-)-epigalocatequina (Arts et al., 2000; de Pascual-Teresa et al., 2000; Määttä-Riihinen et al., 2004; Skupien & Oszmianski, 2004).

Flavonoles	R_1	R_2	R_3
(+)-Catequina	H	OH	H
(-)-Epicatequina	OH	H	H
Galocatequina	H	OH	OH
Epigalocatequina	OH	H	OH
Epicatequina-3-galato	OH	H	Ácido gálico

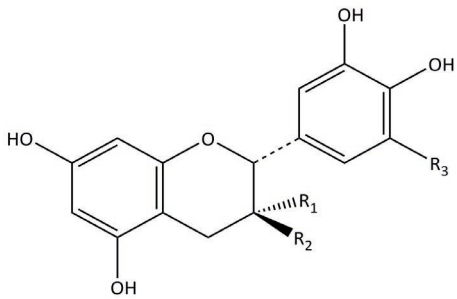


Figura 9:
Estructura química de los flavanoles.

Las procianidinas son químicamente oligómeros de flavanoles, formados a partir de (+)-catequina y (-)-epicatequina unidos entre sí por enlaces C–C. Se han encontrado en fresa procianidinas de tipo dímero como la procianidina B1, B2, B3 y B4 así como otros trímeros de procianidina como la ECC (EC-(4,8)-EC-(4,8)-C) (de Pascual-Teresa et al., 2000).

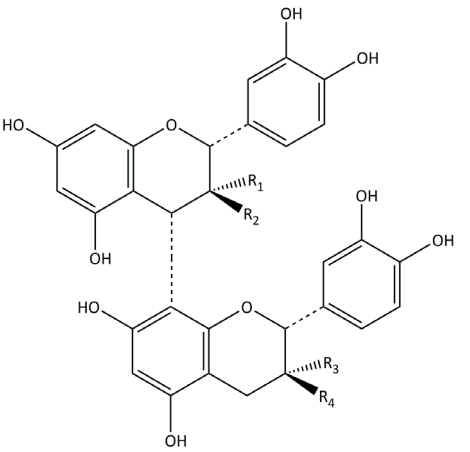


Figura 10:
Estructura química de las procianidinas.

Procianidinas	R_1	R_2	R_3	R_4
Procianidina B_1	OH	H	H	OH
Procianidina B_2	OH	H	OH	H
Procianidina B_3	H	OH	H	OH
Procianidina B_4	H	OH	OH	H

1.2.2.3. Antocianidinas

Las antocianidinas son un grupo de pigmentos hidrosolubles, presentes en las vacuolas de las células vegetales. Se encuentran ampliamente distribuidos en el reino vegetal y son responsables del color azul, morado y rojo en hojas, flores y frutos (Fennema, 1993).
La estructura química de estas agliconas es el ión flavilio el cual funciona como un catión y al cual también se le denomina 2-fenilbenzopirilio y que consta de dos grupos aromáticos: un benzopirilio (A) y un anillo fenólico (B) (Strack & Wray, 1994).

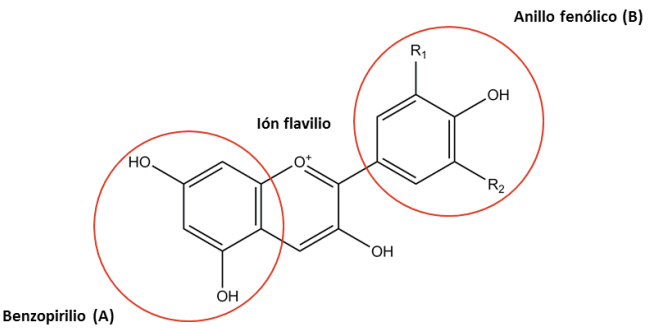


Figura 11: Estructura química de las antocianidinas.

Químicamente las antocianinas son glicósidos de las antocianidinas, es decir, están constituidas por una molécula de antocianidina (aglicona), a la que se le une un azúcar por medio de un enlace β-glicosídico.
Por ejemplo la pelargonidina 3-glucósido (antocianina mayoritaria en la fresa).

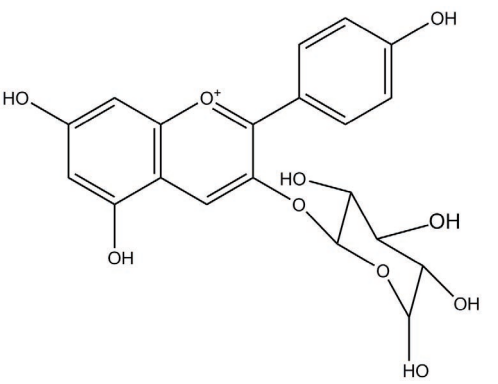


Figura 12: Estructura química de la pelargonidina 3-glucósido.

Las antocianidinas se clasifican en función de:

1. Los sustituyentes en el anillo B. Los seis tipos más comunes se recogen en la siguiente tabla (Tabla 2)

Tabla 2. Tipos más comunes de antocianidinas.

R_1	R_2	Antocianidina
H	H	Pelargonidina
OH	H	Cianidina
OH	OH	Delfinidina
OCH_3	H	Peonidina
OCH_3	OH	Petunidina
OCH_3	OCH_3	Malvidina

2. Número y posición de los azúcares

La glicosilación ocurre principalmente en la posición 3 del anillo C o en las posiciones 5 y 7 del anillo A. También ocurre aunque es menos frecuente en las posiciones 3´, 4´ y 5´del anillo B. Según el número de azúcares presentes en su estructura las antocianinas se clasifican en: monoglicósidos, diglicósidos y triglicósidos. Estos azúcares pueden ser: monosacáridos, disacáridos o trisacáridos. Los monosacáridos más comunes son: pentosas como la arabinosa y la xilosa, o hexosas, siendo la glucosa la más frecuente, también pueden encontrarse galactosa, ramnosa o arabinosa. Los disacáridos más frecuentes

son rutinosa, soforosa, sambubiosa y gentobiosa. Y dentro de los trisacáridos podemos encontrar algunos como la gentotriosa (lineales) o la xilosilrutinosa o glucosilrutinosa (ramificados) (Strack & Wray, 1989).

3. Acilación a los azúcares

Por otro lado, es muy común que estos azúcares estén acilados con ácidos cinámicos (*p*-cumárico, cafeico, ferúlico y sinápico) o ácidos alifáticos (acético, málico, malónico, oxálico y succínico).

Así, si la molécula de antocianidina se encuentra únicamente unida a azúcares, se denominan no aciladas y si además de los azúcares están presentes uno o varios radicales acilo, se catalogan como aciladas. El número de acilantes puede variar de cero a tres y estos pueden ser alifáticos y aromáticos.

1.3 Antocianos de la fresa y factores que afectan a su estabilidad

Los antocianos son los compuestos bioactivos presentes en mayor cantidad en la fresa y las cantidades dependen de la variedad. Raramente aparecen antocianidinas en alimentos (agliconas) estando en la mayoría de los casos como glicósidos.

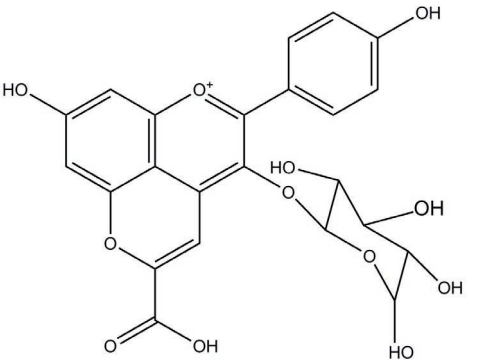


Figura 13: Estructura química de la carboxipiranopelargonidina 3-glucósido.

Los valores totales de antocianos varían entre 150-600 mg/Kg de peso fresco (Clifford, 2000). La pelargonidina 3-glucósido es el antociano mayoritario en la fresa seguido de la pelargonidina 3-rutinósido y de la cianidina 3-glucósido (Lopes-da-Silva et al., 2002; Wu & Prior, 2005). Además la fresa presenta otros antocianos es menores cantidades como la pelargonidina 3-arabinósido y la cianidina 3-rutinósido.

Es muy frecuente la aparición de antocianos acilados habiendo sido identificados entre otros la pelargonidina 3-(6''-malonil-glucósido), pelargonidina 3-O-(6''-succinil-glucósido) o la cianidina-O-(6''-succinil-glucósido) (Wang et al., 2002; Määttä-Riihinen et al., 2004; Skupien & Oszmianski, 2004).
Por otro lado, también se ha identificado en fresa compuestos de tipo piranoantocianos, los cuales se caracterizan por poseer un anillo pirano adicional. Los piranoantocianos son unos pigmentos antociánicos no decolorables y bastante estables ante variaciones de pH, ya que tienen bloqueada la posición C-4 del anillo pirano flavonoideo.
A continuación se presenta la composición polifenólica de la fresa (Tabla 3).

Compuestos fenólicos	Nombre	mg/Kg peso fresco	Referencias
Antocianos y antocianidinas	Cianidina	0,50	Määttä-Riihinen et al., 2004
	Cianidina 3-O-(6''-succinil-glucósido)	1,22	Wang et al., 2002
	Cianidina 3-O-glucósido	2,88	Määttä-Riihinen et al., 2004; Wang et al., 2002; Skupien & Oszmianski, 2004
	Pelargonidina	4,31	Skupien & Oszmianski, 2004
	Pelargonidina 3-O-(6''-malonil-glucósido)	4,78	Määttä-Riihinen et al., 2004
	Pelargonidina 3-O-(6''-succinil-glucósido)	10,44	Määttä-Riihinen et al., 2004; Wang et al., 2002
	Pelargonidina 3-O-arabinósido	0,42	Skupien & Oszmianski, 2004
	Pelargonidina 3-O-glucósido	47,14	Määttä-Riihinen et al., 2004; Wang, et al., 2002; Skupien & Oszmianski, 2004
Flavanoles			
	(+)-Catequina	6,36	Arts et al., 2000; de Pascual-Teresa et al., 2000; Määttä-Riihinen et al., 2004; Skupien & Oszmianski, 2004
	(+)-Galocatequina	0,05	Arts et al., 2000; de Pascual-Teresa et al., 2000
	(-)-Epicatequina	0,0075	Arts et al., 2000; de Pascual-Teresa et al., 2000; Määttä-Riihinen et al., 2004
	(-)-Epicatequina 3-galato	0,28	Arts et al., 2000; de Pascual-Teresa et al., 2000
	(-)-Epigalocatequina	0,06	Arts et al., 2000; de Pascual-Teresa et al., 2000
	Procianidina dímero B1	0,62	de Pascual-Teresa et al., 2000
	Procianidina dímero B2	0,03	de Pascual-Teresa et al., 2000; Määttä-Riihinen et al., 2004
	Procianidina dímero B3	1,10	de Pascual-Teresa et al., 2000
	Procianidina dímero B4	0,13	de Pascual-Teresa et al., 2000
	Procianidina trímero EEC	0,50	de Pascual-Teresa et al., 2000
Flavonoles			
	Kaempferol 3-O-glucósido	0,32	Wang et al., 2002
	Kaempferol 3-O-glucuronido	0,20	Wang et al., 2002
	Morina	0,06	Ehala et al., 2005
	Quercetina 3-O-glucósido	1,74	Määttä-Riihinen et al., 2004
Ácidos fenólicos			
Ácidos hidroxibenzoicos	Ácido 4-hidroxibenzoico 4-O-glucósido	1,53	Schuster & Herrmann, 1985
	Ácido 5-O galoilquínico	0,05	Schuster & Herrmann, 1985
	Ácido elágico	1,24	Mattila & Kumpulainen, 2002; Määttä-Riihinen et al., 2004; Cordenunsi et al., 2002, 2005; Wang et al., 2002;
	Ácido elágico glucósido	2,85	Wang et al., 2002
Ácidos hidroxicinámicos	Ácido 5-cafeoilquínico	1,93	Schuster & Herrmann, 1985; Mattila & Kumpulainen, 2002
	Ácido cafeoil glucósido	0,10	Schuster & Herrmann, 1985
	Ácido cinámico	0,22	Mattila & Kumpulainen, 2002; Ehala et al., 2005
	Ácido feruloil glucósido	0,10	Schuster & Herrmann, 1985
	Ácido *p*-cumárico	0,21	Mattila & Kumpulainen, 2002; Ehala et al., 2005; Määttä-Riihinen et al., 2004
	Ácido *p*-cumárico 4-O-glucósido	0,15	Schuster & Herrmann, 1985; Määttä-Riihinen et al., 2004
	Ácido p-cumaroil glucósido	4,36	Wang et al., 2002
Estilbenos			
	Resveratrol	0,35	Ehala et al., 2005

Tabla 3: Composición polifenólica de la fresa.

El conocimiento de los mecanismos de degradación y de los factores que rigen la estabilidad de los antocianos resulta de gran importancia con el objetivo de evitar pérdidas de estos compuestos y optimizar tanto los procesos de extracción como los procesos tecnológicos a los que son sometidos los alimentos para evitar su deterioro y obtener un color adecuado y estable. A continuación se detallan algunos de los factores que influyen en la composición antociánica de la fresa así como los factores que afectan a su estabilidad general.

1.3.1. Variedad

Se ha comprobado que la cantidad de antocianos varía enormemente entre diferentes variedades de fresa. Del análisis de 8 variedades de fresa se mostró que los antocianos oscilaban entre 20,2-47,4 mg/100 g de peso fresco (Buendía et al., 2010). Asimismo, mayores rangos de variación en las cantidades encontradas de antocianos totales fueron descritos tras el análisis de 27 variedades de fresa distintas (8,5-65,9 mg/100 g (Aaby et al., 2012). Se ha descrito que la variedad *Camarosa* y *Tudnew* son dos de las variedades que poseen mayores concentraciones de antocianos (480-525 mg/100 g de peso fresco) (Lopes-da-Silva et al., 2007).

1.3.2. Estructura química

La estructura química de los antocianos condiciona su estabilidad. La presencia y posición de los grupos hidroxilo y metoxilo, azúcares así como tipo y grado de acilación influyen sobre la estabilidad de estos compuestos (Cabrita et al., 2000; Cooper-Driver, 2001).

Diversos autores han comprobado que los antocianos diglucósidos son más estables que los monoglucósidos a la decoloración ocasionada por efecto del calor y de la luz. Se ha confirmado que la sustitución en C5 reduce el carácter nucleofílico de las posiciones C6 y C8, por ello los antocianos con estructura 3,5–diglucósidos son menos susceptibles al ataque electrofílico que los 3-glucósidos (Shenoy, 1993; Martí et al., 2001).

Por otro lado, la acilación estabiliza los antocianos vía copigmentación intramolecular que a través de estructuras plegadas protegen a los grupos acilo del anillo benzopirilio (A) (Dangles et al., 1993; Jackman & Smith, 1996). La existencia de acilación además potencia la intensidad en el color de las formas quinoidales (Fossen et al., 1998).

1.3.3. pH

Los antocianos son mucho más estables a pH ácido que a pH neutro o alcalino. En medio ácido la forma predominante es el ión flavilio (es deficiente en electrones y, por lo tanto, muy reactivo) que presenta color rojo. Cuando el pH se hace más alcalino este ión es susceptible al ataque nucleofílico por parte del agua dando lugar a la base carbinol (incolora) que darán lugar a las chalconas (amarillas). La expresión máxima de color rojo en los antocianos es a pH 1, cuando las moléculas del pigmento están principalmente en la forma no ionizada.

Generalmente los antocianos en disoluciones acuosas tienen propensión a decolorarse rápidamente e incluso a perder el color completamente (Jackman & Smith, 1996; Clifford, 2000). Por tanto, el pH del medio, al hacer variar la estructura de un antociano, tiene una marcada influencia sobre su color y estabilidad.

En general, los antocianos diglicósidos son más resistentes a la degradación a pH menos ácido que los monoglicósidos (Zafrilla, 1999).

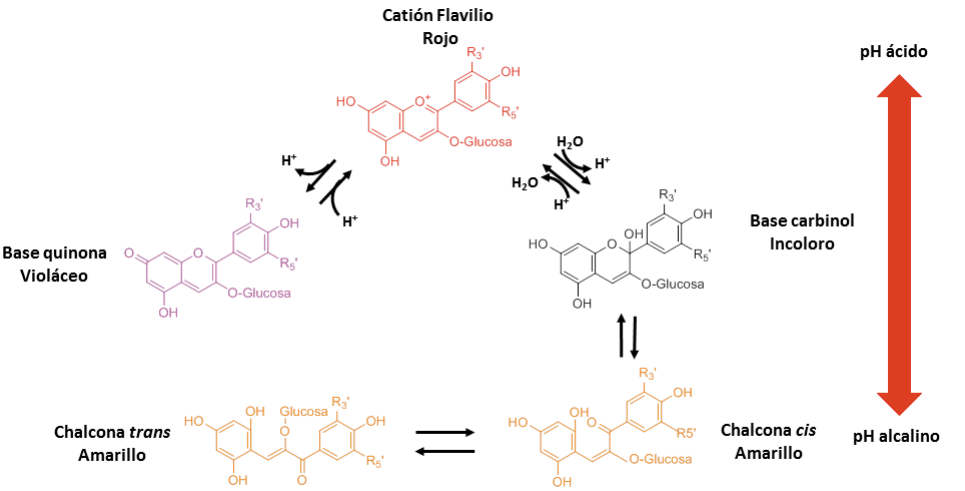


Figura 14: Equilibrio de las antocianinas según el pH (Wong, 1995).

1.3.4. Temperatura

La estabilidad de los antocianos está fuertemente influenciada por la temperatura (Jackman & Smith, 1996). El efecto depende tanto de la magnitud como de la duración de la aplicación.

El incremento de temperatura da lugar a la pérdida del azúcar glicosilante en la posición 3 de la molécula y apertura del anillo benzopirilio con la consecuente producción de chalconas incoloras (Timberlake, 1980). El aumento de la temperatura conlleva una destrucción de los antocianos siendo esta de tipo logarítmica (Delgado-Vargas et al., 2000).

Existen diversas técnicas de calentamiento como secado, microondas, autoclavado, inmersión en agua, pasteurización o escaldado. Sadilova et al. (2006), observaron que el 50 % de los antocianos se destruyen tras 3 horas a 95 ºC. Por el contrario, el tratamiento a temperaturas altas durante tiempos cortos, como el escaldado, no conlleva, en general, una degradación significativa de los antocianos ni transformaciones a especies incoloras (Jackman & Smith, 1996).

Hay estudios que indican que durante la esterilización de algunos frutos rojos, la pérdida de pigmentos es despreciable comparada con la que ocurre durante un enfriamiento lento o el almacenamiento prolongado a temperatura ambiente (Shenoy, 1993).

Asimismo, la metoxilación, la glucosilación y la acilación confieren mayor estabilidad frente a pH y a calentamiento térmico (Jackman & Smith, 1996).
Además, se ha demostrado que la cianidina 3-rutinósido es el antociano que presenta mayor estabilidad a la temperatura (Rubinskiene et al., 2005).

1.3.5. Luz

La luz es otro factor que acelera la degradación de los antocianos. Los antocianos son, en general, más inestables cuando están expuestos a la luz ultravioleta, visible o radiación ionizante (Delgado-Vargas et al., 2000). La luz además, aumenta la tasa de degradación térmica de los antocianos por formación de un estadío excitado del catión flavilio (Furtado et al., 1993).
La formas diglicósidos de los antocianos son más estables frente a la luz que los monoglicósidos y éstas más que sus correspondientes antocianidinas (Delgado-Vargas et al., 2000).

1.3.6. Procesado

Durante el pelado, corte, triturado, etc se produce la rotura de las paredes vegetales y, además, se permite la toma de contacto con diversas enzimas (glucosidasas, polifenoloxidasas y peroxidasas). Este hecho puede provocar la pérdida del azúcar en la posición C3 lo cual, como ya se ha descrito anteriormente, conlleva la apertura del anillo benzopirilio con la consiguiente producción de las formas chalconas (incoloras) (Clifford, 2000).
La pasteurización es otro de los procesos que afecta considerablemente al contenido antociánico. Este proceso conlleva una pérdida de antocianos de aproximadamente un 68 % en zumos de fresa pasteurizados a 100 ºC durante 10 minutos en comparación con el zumo de partida (Narwojsz & Borowska, 2010).
La influencia de los procesos más frecuentemente utilizados en la elaboración de productos derivados de fresa (triturado, puré, zumo y néctar pasteurizado y vino obtenido a partir del zumo o del triturado) en términos de antocianos totales ha sido objeto de estudio, así la producción de zumo y néctar y su posterior pasteurización se tradujo en una pérdida del 44 y 52 %, respectivamente. No obstante, las pérdidas tras la producción de vino resultaron mucho mayores variando ente un 74,4-82,8 % (Klopotek et al., 2005).
Se ha demostrado además que la adición de azúcar a licores de fresa almacenados a 15 ºC preserva los antocianos durante 3 meses. Sin embargo, a 30 ºC y con un almacenamiento de 6 meses se llega casi a la pérdida total de antocianos (Sokól-Lętowska et al., 2014).
La elaboración de mermeladas a partir de fresa produce una pérdida muy acusada de antocianos totales; Rababah et al. (2011) demostraron una pérdida del 96,6 % en comparación con la fresa de partida. El almacenamiento de esta mermelada durante 5 meses también conllevó a una pérdida adicional del 11,5 %.
Se ha descrito una pérdida de entre el 63-85 % tras la fermentación alcohólica de fresa, siendo esta pérdida aún más acusada tras la fermentación acética en comparación con el sustrato de partida (97,2-99 %) (Ubeda et al., 2013). La fermentación acética de otros tipos de frutas como la granada, en concordancia con otros estudios conlleva a una pérdida de antocianos totales del 90,2 % (Orduodi et al., 2014).

1.4. Otros compuestos bioactivos presentes en la fresa y en alimentos fermentados

Además de los compuestos polifenólicos cada vez va adquiriendo mayor importancia la identificación de otros compuestos bioactivos presentes tanto en vegetales como en alimentos fermentados.

Entre ellos la melatonina (N-acetil-5-metoxitriptamina) ha sido y es uno de los compuestos más estudiados, gracias a sus ya demostrados efectos antioxidantes (Hardeland et al., 1993, 1995; Reiter et al., 2000; Okatani et al., 2002; Mayo et al., 2002, 2003a, 2003b; Allegra et al., 2003; Rodriguez et al., 2004), antienvejecimiento (Reiter, 1995) y antitumorales (Guerrero et al., 2007).

La melatonina es una neurohormona que es sintetizada principalmente por la glándula pineal de los mamíferos a partir del aminoácido triptófano y está relacionada con los ciclos de luz/oscuridad. Químicamente presenta un anillo de tipo indólico y dos grupos funcionales principales, el grupo metoxi en posición 5 y el grupo amida en posición 3 (Posmyk & Janas, 2009). La presencia de estos grupos determina su solubilidad en medio acuoso y orgánico, gracias a su carácter anfipático puede atravesar las membranas celulares y ser transportada por fluidos biológicos (Hardeland et al., 2006).

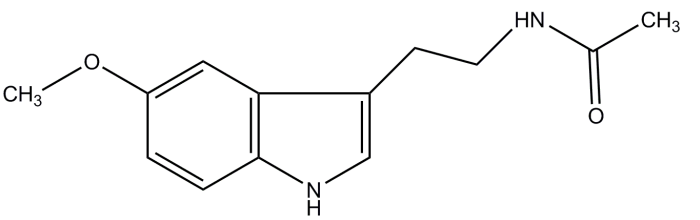


Figura 15: Estructura química de la melatonina.

Los niveles de melatonina son mayores en personas jóvenes y van disminuyendo gradualmente con la edad (Cardinali et al., 2008). Se ha encontrado melatonina en hojas, frutos y semillas de diversas plantas cuyas concentraciones varían y se encuentran en orden de picogramos o nanogramos por gramo (Burkhardt et al., 2001; Reiter et al., 2005; de la Puerta et al., 2007; Paredes et al., 2009).

En cuanto a la fresa se refiere, los niveles encontrados varían entre 1.4-134.6 pg/g según la variedad (Hattori et al., 1995; Badria, 2002; Stürtz et al., 2011) (Tabla 5).

Tabla 4: Concentraciones de melatonina en distintas variedades de fresa.

Fresa (variedad)	Concentración	Referencia
Magna	12,4-134,6 pg/g	Hattori et al., 1995 Badria, 2002
Camarosa	1,4-5,58 pg/g	Stürtz et al., 2011
Candonga	2,1-5,5 pg/g	Stürtz et al., 2011
Festival	3,28-11,26 pg/g	Stürtz et al., 2011
Primoris	4,2- 8,5 pg/g	Stürtz et al., 2011

Además la melatonina ha sido identificada en vinos y en otras bebidas fermentadas en concentraciones que oscilan entre 0,16-9880 ng/mL. Su presencia en vinos ha sido asociada al metabolismo de varias cepas de *Saccharomyces* durante la fermentación alcohólica (Rodriguez-Naranjo et al., 2011).

Se han identificado asimismo otros compuestos derivados de la síntesis o intermediarios del metabolismo de la melatonina (Figura 16) en alimentos fermentados. Se han encontrados cantidades relevantes de serotonina, del orden de mg/L tanto en cerveza como en vino (Kirschbaum et al., 1999; Bartolomé et al,.2000, Manfroi et al., 2009; Wang et al., 2014).

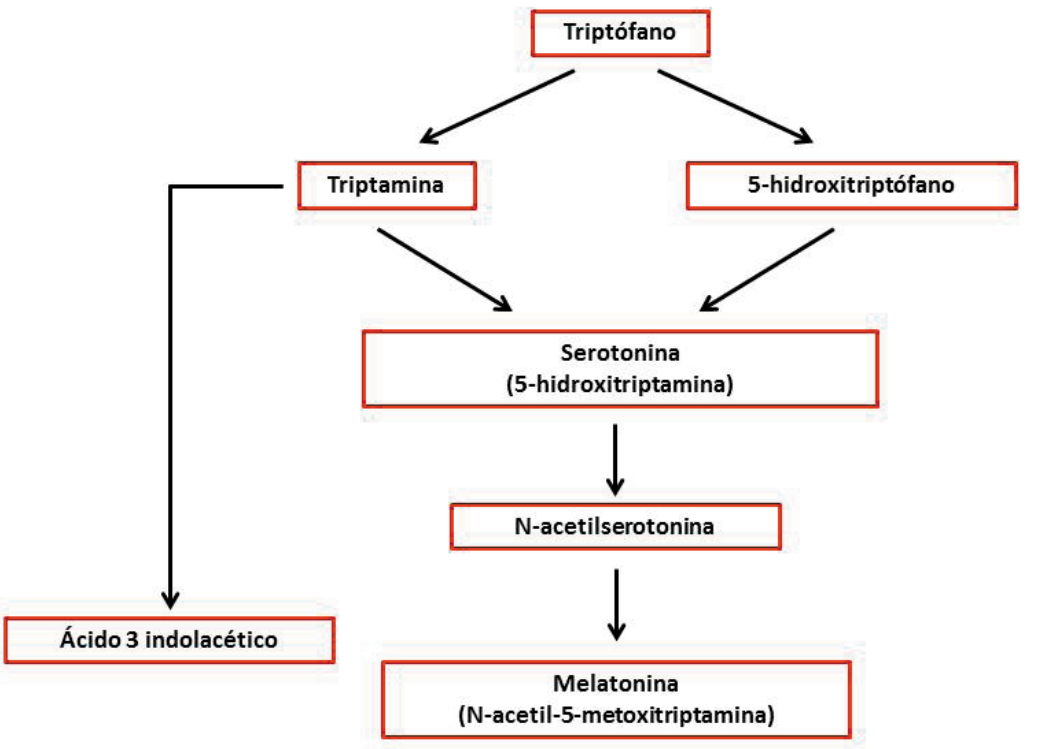


Figura 16:
Ruta biosintética de la melatonina (incluyendo mamíferos, aves y anfibios) (Reiter, 1991; Falcón et al., 2009)

1.5. Biodisponibilidad de los antocianos

La biodisponibilidad es un concepto que engloba las distintas fases que determinan el grado de utilización biológica de un fármaco, nutriente o bioactivo: liberación de la matriz, absorción, distribución, metabolismo y excreción (Aggett, 2010; Hurrel & Egli, 2010).
Sí bien, en el ámbito farmacológico, la liberación de los principios de la forma farmaceútica la cual determina su formulación ha sido objeto de numerosos estudios, en el caso de los alimentos es un campo más reciente e inexplorado.

El proceso de digestión comienza en la boca, la acción de las enzimas de la microbiota presentes, la temperatura y la unión de los antocianos a proteínas de la saliva puede provocar un descenso de estos compuestos (Kamonpatana et al., 2012). Asimismo, se llevan a cabo algunas desglicosilaciones que dan lugar a agliconas (Mallery et al., 2011). El tiempo que el alimento se encuentra en la cavidad oral es relativamente corto, por ello muchos autores no tienen en cuenta los procesos que ya se producen en la boca y describen las distintas trasformaciones que sufren los antocianos a partir de su llegada al estómago.

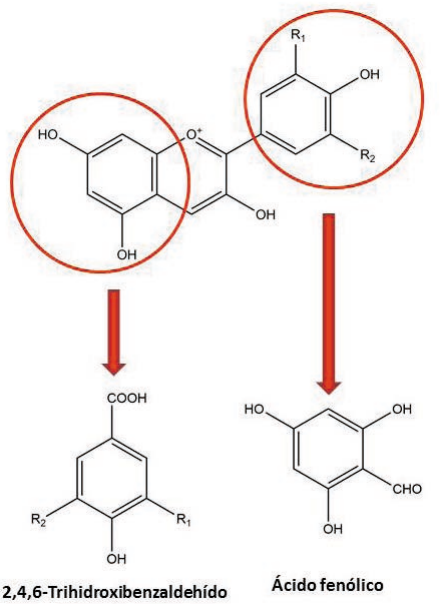
La absorción de los antocianos tiene lugar principalmente en el estómago e intestino delgado (ID). Se ha descrito absorción de los antocianos en el estómago ya que se ha demostrado que estos aparecen rápidamente en plasma tras su consumo (Milbury et al., 2002; Passamonti et al., 2003; Talavera et al., 2005). Parece ser que la barrera gástrica permite el paso de los antocianos al torrente circulatorio gracias a la acción de la bilitranslocasa, proteína de membrana capaz de transportar aniones orgánicos solubles en agua (Passamonti et al., 2002).

Ya en el ID, ocurre la hidrólisis de los glicósidos formando las correspondientes agliconas más hidrofóbicas, las cuales pasan a los enterocitos mediante difusión pasiva.
Los glicósidos, sin embargo, requieren un transporte activo (Kay, 2006). Parece que el transporte de los antocianos a través de las células del epitelio intestinal se lleva a cabo mediante varios transportadores de glucosa como el GLUT2 y el SGLT1 (Faria et al., 2009; Walton et al., 2006).
Debido al pH alcalino intestinal, los antocianos se encuentran mayoritariamente en forma de carbinol pseudobase con lo cual su absorción se ve más limitada (Crozier et al., 2009). La absorción de los antocianos depende además de la región del ID: se ha descrito un 55,3±7,6 % en el yeyuno; 10,4±7,6 % en el duodeno y no se ha observado en el íleo (Matuschek et al., 2006). Aproximadamente el 7,5 % del total de la ingesta de antocianos se encuentran en el ID en su forma original (inalterado) tras 2 horas de administración de frambuesa en ratas (He et al., 2009).
Además, también se han encontrado en yeyuno tras la administración de una dieta rica en antocianos durante 15 días, cianidina 3-glucósido y sus conjugados correspondientes (metilados, glucurónidos) (Talavera et al., 2005).
Los antocianos en los tejidos gastrointestinales pueden llegar a alcanzar concentraciones del orden de µM., no obstante, en sangre estas concentraciones disminuyen considerablemente. Estas concentraciones son muy similares a las usadas en diversos estudios *in vitro* para

comprobar los efectos beneficiosos de estos compuestos (Basu et al., 2010a; Tsuda, 2012; Chen & Chen, 2013). Durante su metabolismo los antocianos sufren reacciones de conjugación en las células hepáticas y renales (metilación, sulfatación y glucuronización). En general, los compuestos que llegan a la sangre y a los tejidos son estas formas conjugadas y las formas intactas. Estudios más recientes han demostrado que los antocianos no absorbidos en el ID pasan al intestino grueso y son sometidos a la acción de la microbiota colónica. La microbiota ejerce un papel muy importante en el metabolismo, considerándose recientemente como un "órgano metabólico" en sí mismo.

En el colon los antocianos se encuentran a un pH que propicia su inestabilidad, así a los 20 minutos de incubación de los antocianos con la microbiota intestinal aparecen los primeros productos de degradación que son los ácidos fenólicos correspondientes al anillo B y el 2,4,6-trihidroxibenzaldehído (aldehído de floroglucinol) correspondiente al anillo A (Keppler & Humpf, 2005) (Figura 17).

Figura 17:
Productos de degradación de los antocianos por acción de la microbiota intestinal.



Se ha descrito un rápido descenso del antociano mayoritario, la malvidina 3-glucósido así como de sus formas aciladas, llegándose a la degradación completa de los antocianos a las 30 horas de incubación de un extracto de vino tinto con microbiota fecal humana (Sánchez-Patán et al., 2012). Dentro de los productos de degradación de los antocianos, se han encontrado el ácido protocatéquico, siríngico y vainíllico.
Además los ácidos siríngico y vainíllico pueden ser desmetilados por la flora intestinal y formar ácido gálico y ácido protocatéquico, respectivamente.

Inicialmente, se puso en evidencia que el ácido protocatéquico es el metabolito mayoritario por efecto de la microbiota colónica sobre la cianidina 3-glucósido y su aglicona tras incubación *in vitro* (Aura et al., 2005). Posteriormente, este metabolito ha sido identificado en heces después de 6 horas de consumo de una fuente rica en cianidina 3-glucósido (Vitaglione et al., 2007). Asimismo, tras el consumo de fresas (750 g) se han encontrado en orina tras 5 horas los ácidos 4-hidroxibenzoico, ácido protocatéquico, vainíllico y gentísico (Russel et al., 2009).

A su vez los metabolitos formados por la acción de la microbiota pueden ser absorbidos y metabolizados (Kay et al., 2009).
Mediante el uso de células hCEMEC/D3, modelo utilizado como Barrera Hematoencefálica (BHE) humana, se ha comprobado que los metabolitos de los antocianos muestran mejor eficiencia en el transporte que sus antocianos originales (Fernandes et al., 2014).
Asimismo, se ha encontrado cianidina 3-glucósido en concentraciones de 0.21 nmol/g en cerebro de ratas alimentadas durante 15 días con una dieta enriquecida en antocianos a partir de moras (Talavera et al., 2005). También han sido identificados: cianidina 3-galactósido, cianidina 3-glucósido, cianidina 3-arabinósido, malvidina 3-galactósido, peonidina 3-arabinósido y delfinidina 3-galactósido en cerebro de ratas alimentadas con una dieta que contenía un 2 % de arándanos durante 10 semanas (Andres-Lacueva et al., 2005).
Además se han identificado 11 antocianos en su estructura intacta en hígado, ojo, cortex y cerebelo. Por otra parte, ninguno de ellos ha sido descrito ni en orina ni en plasma de cerdos suplementados durante 4 semanas con arándanos y sometidos a un periodo de entre 18-21 horas de ayuno antes de la eutanasia (Kalt et al., 2008). Este hecho sugiere que los antocianos se distribuyen y quedan retenidos en los tejidos aunque el mecanismo de retención no está aun claramente dilucidado.

Las concentraciones de ácido protocatéquico en plasma varían entre 0,2-2 µM tras la digestión gastrointestinal y su posterior degradación por parte de la microbiota tras el consumo de 500 mg de cianidina 3-glucósido (Czank et al., 2013; de Ferrars et al., 2014). Además, este ácido fenólico ha sido identificado en cerebro de rata, demostrándose que este compuesto es capaz de atravesar la BHE (Zhang et al., 2011).

La gran mayoría de los estudios han establecido que los antocianos procedentes de distintos alimentos se absorben rápidamente alcanzándose concentraciones en plasma (C_{max}) entre 1,4 y 592 nmol/L tras la ingestión de dosis de antocianos de entre 56 y 1300 mg, en rangos de tiempo de entre 20 minutos y 4 horas y con excreciones en orina de entre el 0,003-5,10 % del total de antocianos ingerido.
La excreción máxima tiene lugar entre las 1 y 4 horas después del consumo (Lapidot et al., 1998; Cao & Prior, 1999; Netzel et al., 2001; Mülleder et al., 2002; Mazza et al., 2002; Wu et al., 2002; Milbury et al., 2002; Nielsen et al., 2003; Frank et al., 2003; McGhie et al., 2003; Felgines et al., 2003, 2005; Kay et al., 2004; Garcia-Alonso et al., 2009).

Particularmente, en estudios realizados en voluntarios humanos tras el consumo de fresa (entre 100-400 g) la excreción urinaria (antocianos y sus metabolitos), expresados como % de la ingesta varía entre 1-2,35 % a las 24 horas (Felgines et al., 2003; Mullen et al., 2008; Carkeet et al., 2008; Hollands et al., 2008).

En otro estudio de intervención se identificaron 28 metabolitos de antocianos en orina y 21 en plasma tras el consumo durante 12 semanas de 500 mg de antocianos (mayoritariamente compuesto por cianidina 3-glucósido y cianidina 3-sambubiósido), entre ellos los ácidos vainíllico, ácido protocatéquico y benzoico así como sus formas sulfatadas y glucuronidadas.
Las concentraciones de los metabolitos fenólicos en el plasma fueron de 1237 nM, mientras que las concentración de antocianos conjugados solo alcanzó una concentración de 34 nM en plasma, en orina se encontraron concentraciones de 33185 y 548 nM, respectivamente (de Ferrars et al., 2014)

Debido a la mayoría de los compuestos encontrados en plasma u orina son estos metabolitos de los antocianos, muchos autores coinciden en que los efectos beneficiosos atribuidos a los antocianos pueden ser debidos en parte a la bioactividad de estos compuestos fenólicos.
Por ello, en la presente Tesis Doctoral, se ha evaluado el papel del ácido protocatéquico en el proceso de agregación de diferentes proteínas implicadas en el desarrollo de enfermedades neurodegenerativas, así como su papel neuroprotector frente a la toxicidad de éstas, teniendo en cuenta que es uno de los principales metabolitos de antocianos y que además es un compuesto capaz de atravesar la BHE pudiendo ejercer su efecto a nivel cerebral.

Figura 18: (página siguiente)

1.6. Actividad biológica de los antocianos.

Estudios epidemiológicos asocian el consumo de fresa a efectos protectores que han sido ampliamente revisados (Basu et al., 2014).
Además, numerosos trabajos de intervención con voluntarios humanos han demostrado propiedades beneficiosas al modificarse marcadores biológicamente plausibles relacionados con la hipertensión, oxidación de lipoproteínas, glucosa sanguínea, respuesta inflamatoria o riesgo trombótico (Basu et al., 2009, 2010b; Törrönen et al., 2010; Henning et al., 2010; Burton-Freeman et al., 2010; Ellis et al., 2011) los cuales han sido atribuidos a los compuestos polifenólicos presentes en la fresa, entre ellos los antocianos.
En cuanto a las propiedades neuroprotectoras se ha asociado el consumo de frutas ricas en polifenoles (fresas, arándanos, moras, etc) a la mejora de la memoria y otras capacidades cognitivas (Spencer, 2010; Subash et al., 2014).
Gran parte de estos efectos se basan en la capacidad de estos compuestos para combatir los ROS y actuar como quelante de iones metálicos (Aquilano et al., 2008).

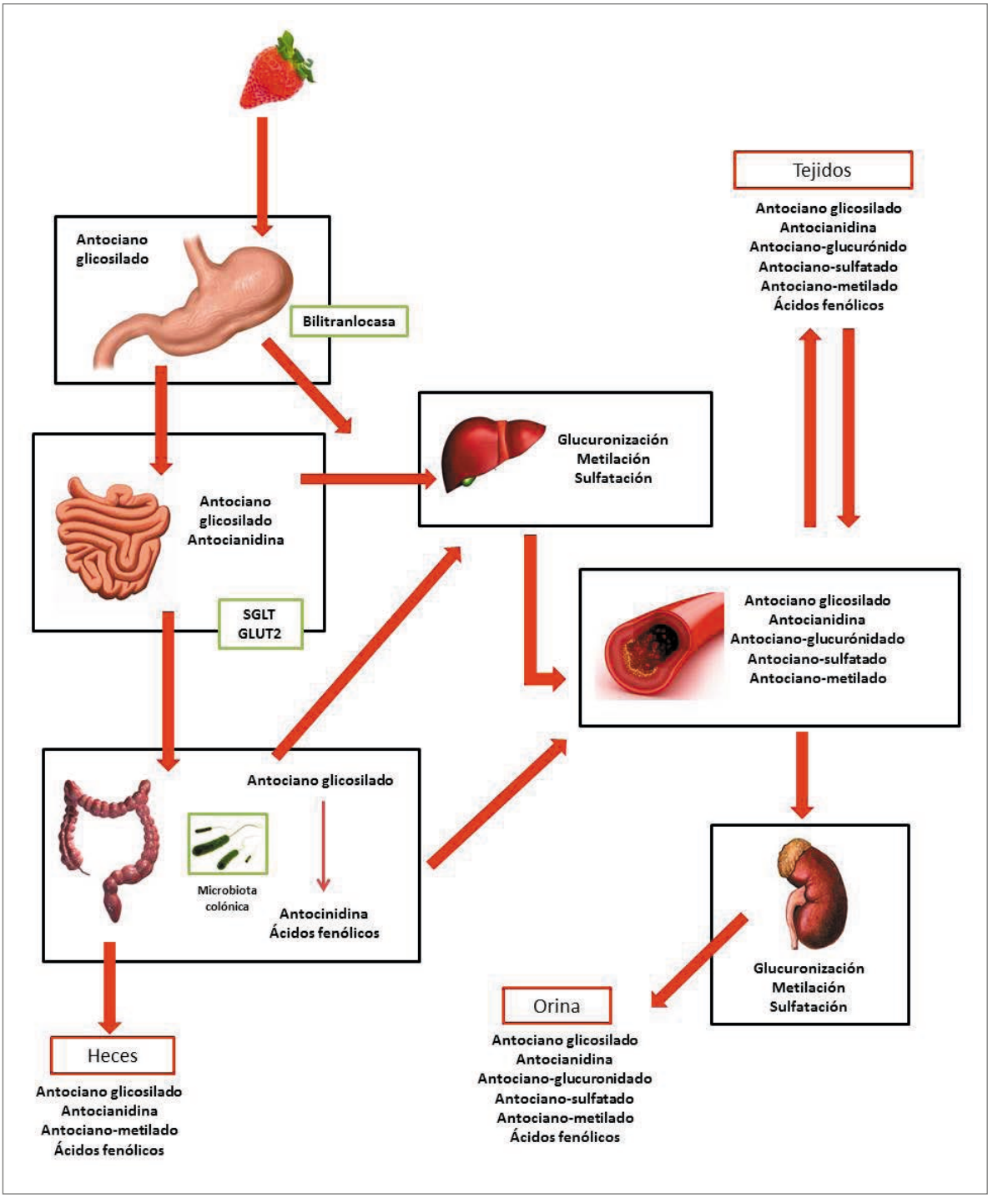


Figura 18: Ruta esquemática de absorción, metabolismo, distribución y eliminación de antocianos en humanos (adaptado de McGhie & Walton, 2007).

Debido a los resultados prometedores obtenidos hasta el momento, se están llevando a cabo cada vez más investigaciones *in vitro* e *in vivo* sobre el papel de los antocianos en la prevención de enfermedades neurodegenerativas que se exponen a continuación.

Se ha comprobado que los antocianos presentes en la patata dulce morada disminuyen la apoptosis producida por el péptido β-amiloide (Aβ) en células PC12 como consecuencia de la disminución de los ROS, la peroxidación lipídica y la activación de la caspasa-3 (Ye et al., 2010). Recientemente, Badshah et al. (2015) demostraron que los compuestos antociánicos procedentes de soja negra previenen de los efectos neurotóxicos producidos por el péptido $A\beta_{1-42}$ normalizando el potencial de membrana mitocondrial y los niveles de calcio, inhibiendo la ruta de apoptosis mitocondrial y disminuyendo la muerte neuronal en células HT22 y en ratas adultas.

Otro estudio ha evidenciado que la cianidina protege de la muerte a células SK-N-SH frente a la toxicidad producida por el Aβ e inhibe la apoptosis celular, resultado de la reducción del estrés oxidativo, la inhibición de la liberación del citocromo C y la activación de las caspasas 9 y 3, las cuales llevan a la atenuación de la apoptosis (Thummayot et al., 2014). Además, Tarozzi et al. (2010) comprobó que la cianidina 3-glucopiranósido es capaz de inhibir la agregación del $A\beta_{25-35}$ y prevenir de la muerte celular en células SH-SY5Y expuestas al efecto tóxico de este péptido.

Asimismo, los antocianos han demostrado *in vivo* efectos favorables en la inflamación, alteración de la comunicación neuronal y en la modulación de las vías de señalización del estrés (Joseph et al., 2009). Estos compuestos actúan inhibiendo las monoaminas oxidasas A y B (enzimas que catalizan la oxidación de monoaminas y degradan los neurotransmisores) y la actividad proteosomal confiriendo por tanto efectos neuroprotectores (Dreiseitel et al., 2009).

Otros estudios *in vivo* han demostrado que extractos ricos en antocianos también atenúan los efectos tóxicos de la rotenona (producción de ROS, activación de microglías e inflamación y agregación de la proteína α-sinucleína (αS)) evitando la muerte de células dopaminérgicas en células primarias como modelo de la Enfermedad de Parkinson (PD) (Strathearn et al., 2014).

Además, se ha comprobado que la administración de pelargonidina atenúa anormalidades del comportamiento, marcadores de estrés oxidativo y protege las neuronas del Sistema Nervioso Central (SNC) frente a toxicidad de la 6-hidroxidopamina (6-ODDA) en ratas modelo de PD (Roghani et al., 2010).

2. Enfermedades neurodegenerativas

Las enfermedades de Alzheimer y de Parkinson son los desórdenes neurodegenerativos con mayor prevalencia cuya incidencia se ve incrementada con la edad. Uno de los mecanismos comunes en el desarrollo de ambas enfermedades es el mal plegamiento y agregación de proteínas que tras un complejo proceso resultan en estructuras fibrilares que se acumulan en diferentes zonas del cerebro y dan lugar al desarrollo y aparición de la sintomatología propia de estos desórdenes. Estos plegamientos anómalos han sido asociados además a la manifestación de otras enfermedades neurodegenerativas (Figura 19).

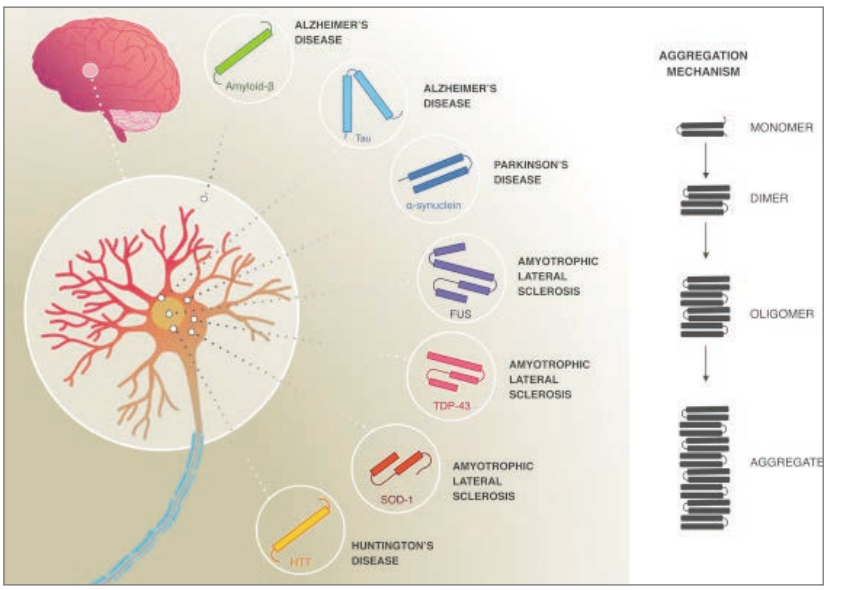


Figura 19:
Enfermedades en las que está implicado el plegamiento anormal de proteínas. Mecanismo de agregación de proteínas en las enfermedades neurodegenerativas. En estos desórdenes los monómeros en su estado nativo se unen adquiriendo una conformación en hoja β-plegada que da lugar a oligómeros y finalmente forman agregados fibrilares altamente ordenados. Este proceso está asociado con la neurodegeneración y produce depósitos proteicos insolubles que causan la muerte celular (de Calignon, 2010).

2.1. Enfermedad de Alzheimer (AD)

La enfermedad de Alzheimer (AD) fue descrita por primera vez en 1907 por el psiquiatra y neurólogo Aloïs Alzheimer.
El Alzheimer es una enfermedad neurodegenerativa e irreversible caracterizada por pérdida de memoria progresiva y graves trastornos de personalidad en las etapas iniciales de la enfermedad, así como por la pérdida de la capacidad cognitiva (afasia, apraxia y agnosia), ansiedad, insomnio, paranoia y en los estadíos más avanzados se va desarrollando la demencia.

Los pacientes manifiestan un deterioro progresivo hasta perder la capacidad de desempeñar una vida autónoma e independiente, quedando en estado vegetativo durante los últimos meses, e incluso años, de su vida. La etiología de la enfermedad es desconocida para el 90 % de los casos, solo un pequeño porcentaje puede ser explicado por causas genéticas.
Las alteraciones macroscópicas observadas en el cerebro de pacientes de AD son: atrofia cortical severa, adelgazamiento de las circunvoluciones y ensanchamiento de los surcos, engrosamiento de las meninges, agrandamiento de las cavidades ventriculares, disminución del bulbo olfatorio y pérdida de peso y volumen cerebral (Halliday et al., 2003).

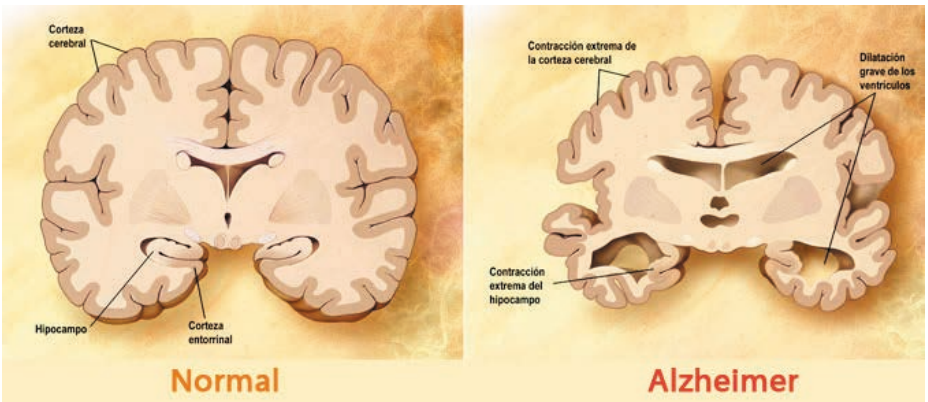


Figura 20:

(Der.) Cerebro afectado por AD (Izq.) Cerebro sano *(http://culturacientifica.com/app/uploads/2013/07/cerebro_corte_frontal_alzheimer.jpg)*

En cuanto a las alteraciones microscópicas características de la AD encontramos: agregados proteicos extracelulares de péptido Aβ que forman placas seniles y los ovillos neurofibrilares intraneuronales de la proteína tau hiperfosforilada (Figura 21), la disminución de la densidad sináptica y la degeneración neuronal en las áreas cerebrales afectadas (Hardy & Selkoe, 2002).

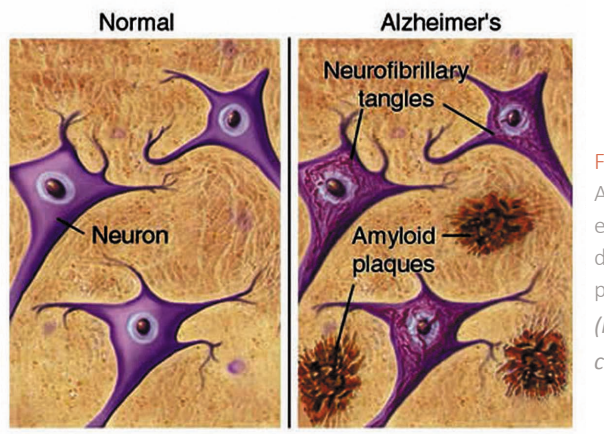


Figura 21:
Alteraciones microscópicas presentes en el cerebro de pacientes con AD. Placas de péptido Aβ y ovillos neurofibrilares de proteína tau.
(https://eduardosetti.files.wordpress.com/2014/10/neurona-con-alzheimer.jpg)

En la actualidad la AD no tiene un tratamiento farmacológico eficaz ya que aún se desconocen las dianas terapeúticas. A mediados de los años 70, se estableció la hipótesis colinérgica de la AD (la cual se comentará posteriormente) que asociaba la mayoría de los síntomas de la enfermedad a la pérdida de un gran número de neuronas colinérgicas (Whitehouse et al., 1981; Arendt et al., 1985). En base a esta hipótesis se comenzaron a utilizar fármacos inhibidores de la acetil colinesterasa como tratamiento de la enfermedad.
Más tarde fue demostrado que aproximadamente en el 50 % de los pacientes tratados con estos fármacos se produce un retraso parcial en el déficit cognitivo. Por ello, el sistema colinérgico no es la única diana terapéutica para combatir la sintomatología de la enfermedad.

2.2. Etiología de la AD

Se distinguen dos tipos de AD, la familiar y la esporádica. La mayoría de casos son esporádicos y aproximadamente entre un 5-10 % corresponde a formas familiares frecuentemente de inicio temprano (Shastry & Gibilin, 1999).

2.2.1. Alzheimer familiar

Se caracteriza por un comienzo temprano y se manifiesta antes de los 60 años.
Representa menos del 5 % de los casos de Alzheimer y se hereda con carácter autosómico dominante.
Está asociada a mutaciones patogénicas en los genes de la proteína precursora del péptido amiloide (APP, en el cromosoma 21), presenilina 1 (PSEN1 en el cromosoma 14q) y presenilina 2 (PSEN2 en el cromosoma 1) (Bertram et al., 2010) entre otras.
Las mutaciones en la APP se encuentran en las zonas de escisión relacionadas con la producción del péptido Aβ. La PSEN1 y la PSEN2 están implicadas en la producción de Aβ.
Asimismo, duplicaciones de la APP también son causantes de AD, como en el Síndrome de Down (trisomía del 21).
Todas las mutaciones tienen como resultado la formación anómala del péptido Aβ produciendo posteriormente placas amiloides (Levy-Lahad et al., 1995; Sherrington et al., 1996; Hardy, 1997).

2.2.2. Alzheimer esporádico

Esta forma de inicio más tardío que la forma familiar, afecta al 3-4 % de las personas de entre 60 y 65 años. A partir de esta edad, la incidencia aumenta progresivamente afectando a más del 40 % de la población a partir de los 80 años.

A pesar de que la forma esporádica no tiene origen genético, se han descrito polimorfismos en ciertos genes asociados a un mayor riesgo de padecer la enfermedad entre ellos el gen que codifica para la Alipoproteína E (APOE). APOE presenta tres isoformas mayoritarias, APOE-2, APOE-3 y APOE-4. Especialmente, APOE-4 está considerado como el mayor factor de riesgo de asociación genética, ya que entre un 65-80 % de los enfermos de AD presentan al menos uno de los dos alelos APOE-4. La presencia de dos alelos de APOE-4 incrementa 10 veces el riesgo de padecer AD con respecto a aquellas personas que carecen de los mismos (Farrer et al., 1997; Eisenstein, 2011).

Asimismo, existen diferencias en la incidencia según el sexo. Así, las mujeres tienen mayor riesgo de padecer la enfermedad que los hombres (Reiman & Caselli, 1999), hecho que puede explicarse debido a la disminución de estrógenos en la menopausia. Ensayos *in vitro* han demostrado la capacidad de los estrógenos para disminuir la producción de Aβ (Turner, 2001) y propiedades neuroprotectoras (Maccioni et al., 2009). Recientemente ha sido descrita una asociación entre problemas cardiovasculares debidos a aterosclerosis, hipertensión, diabetes mellitus o procesos inflamatorios y el riesgo de inicio y desarrollo de la AD (Huang et al., 2014; O´Brien & Markus, 2014; Butterfield et al., 2014).

Además ciertos factores ambientales como la alimentación, la situación económica, el nivel educativo, el entorno o hábitos de vida como el tabaco pueden influir en la predisposición de

2.3. Hipótesis sobre el origen de la AD.

padecer AD de tipo esporádico.
Como ya se ha comentado anteriormente se desconoce la causa de la AD. Existen una serie de hipótesis en base a los cambios moleculares que se producen en la enfermedad, las cuales

1. A la deficiencia del neurotransmisor acetilcolina (Hipótesis Colinérgica)

2. A alteraciones en la proteína tau (Hipótesis de la Proteína tau)

3. A la acumulación asociada al péptido Aβ (Hipótesis de la cascada Amiloide)

2.3.1. Hipótesis Colinérgica

proponen que el origen de la AD se debe:
La Hipótesis Colinérgica relacionada con la pérdida de memoria fue descrita en 1982 por Bartus y sus colaboradores (Bartus et al., 1982).
Esta hipótesis propone la existencia de una alteración de la actividad colinérgica en cerebros de ancianos y en pacientes con demencia debida a la deficiencia del neurotransmisor acetilcolina (ACh). Esta hipótesis se basa en que el tratamiento con inhibidores de la colinesterasa producen efectos positivos sobre las capacidades cognitivas en pacientes con la AD (Hansen et al., 2008).
En condiciones fisiológicas, la ACh es sintetizada en el interior de la neurona a partir de la colina (Ch) por la acción de la enzima acetilcolintranferasa (ChAT).
Una vez sintetizada la ACh se almacena en vesículas en el interior de la neurona presináptica. La ACh es un neurotransmisor y es liberada por las neuronas al espacio sináptico donde ejerce su

2.3.2. Hipótesis de la Proteína tau

función sobre otras neuronas u otras células.
Una de las proteínas que se acumulan de forma anómala en el cerebro de pacientes con AD es la proteína tau. Tau es el componente mayoritario de los ovillos neurofibrilares y se expresa en neuronas del SNC.
El desarrollo o acumulación de formas insolubles e hiperfosforiladas de esta proteína y su posible relación con la AD fue descrito por Brakk y Brakk (1991).
La función celular de la proteína tau es modular la adhesión de los microtúbulos y así contribuir a la estabilización en las neuronas.
La fosforilación anormal de esta proteína en sitios críticos de su estructura, disminuye su capacidad para promover la adhesión de los microtúbulos bloqueando su acción estabilizadora, lo que conllevaría a la degeneración neuronal.
El mecanismo de fosforilación de esta proteína *in vivo* no está claramente dilucidado, se ha demostrado que dos enzimas de tipo quinasas están implicadas en este proceso (TPK I y TPK II) (Imahori & Uchida, 1997; Imahori et al., 1998). La inhibición de estas enzimas es un objetivo terapéutico de interés.

2.3.3. Hipótesis de la Cascada Amiloide

Esta hipótesis es la más aceptada en la actualidad. En ésta, se postula que el origen de la AD esporádica se debe a un exceso y acumulación anómala de proteínas β amiloides en el exterior de las neuronas y a la neurotoxicidad de estos agregados resultante de un desequilibrio entre su producción y su eliminación.
Esto podría desencadenar una cascada cuyo resultado final son los síntomas relacionados con la AD (Hardy & Selkoe, 2002).

Esta hipótesis se ve apoyada ya que en los casos de AD de origen genético como ya se ha comentado con anterioridad, las mutaciones genéticas encontradas (APP, PSEN1, PSEN2)

aparecen asociadas a proteínas implicadas en la génesis de Aβ que además cursan con depósito excesivo de Aβ.
A continuación se presenta un esquema de las distintas fases en las que se basa esta hipótesis (Figura 22).

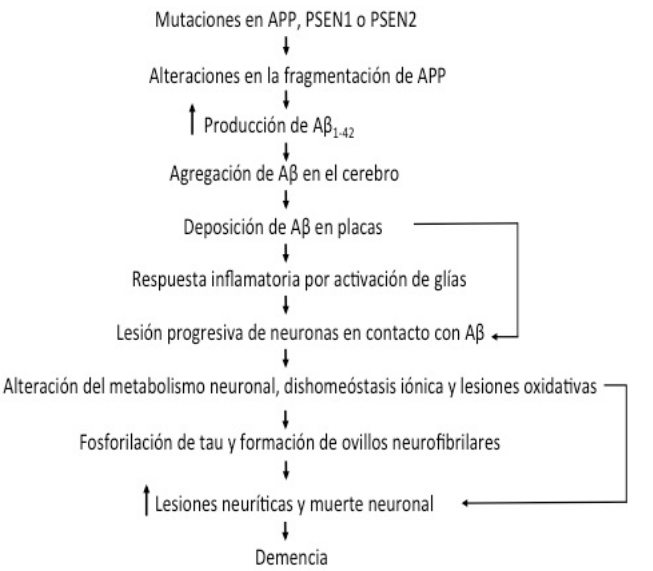


Figura 22: Hipótesis de la Cascada Amiloide en la AD. En la AD existe un aumento de la producción y acumulación excesiva de Aβ1-42 en el cerebro, lo que conlleva alteraciones como inflamación, dishomeóstasis iónica, daño oxidativo y aumento de tau que desemboca finalmente en neurodegeneración y demencia (Hardy & Selkoe, 2002).

2.4. Péptido Aβ

El péptido Aβ contiene entre 39 y 43 aminoácidos y es un producto derivado de la APP (De Strooper et al., 2010).
In vivo existen dos formas mayoritarias de este péptido: la Aβ40 que termina en el aminoácido Valina 40 (Val40) y la Aβ42 con dos aminoácidos más, Isoleucina 41 (Ile41) y Alanina 42 (Ala42). El Aβ42 posee mayor hidrofobicidad y capacidad de agregación (Wolfe, 2007).
Estas características hacen que el Aβ42 sea más abundante en las placas que el Aβ40.

El péptido Aβ se forma a partir de la APP (Figura 23). La proteína APP es una proteína de membrana que tras su síntesis se procesa.
Este procesamiento puede ocurrir a través de varias vías. La APP puede sufrir diversas escisiones proteolíticas por la actividad de las secretasas α-, β- y γ-. La proteína APP será procesada por la vía no amiloidogénica (no patológica, normal) o amiloidogénica (patológica) dependiendo de la enzima que actúe.

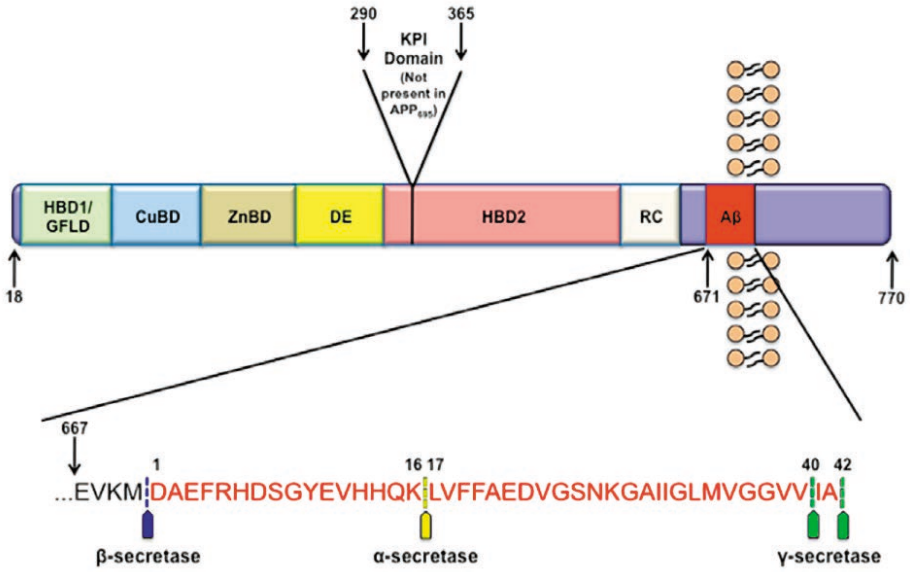


Figura 23: Zonas de escisión de las distintas secretasas en el procesamiento de la APP. (Lazarov & Demars, 2012)

2.4.1. Vía no amiloidogénica

La primera enzima implicada en esta vía es la α-secretasa. Esta enzima escinde la proteína APP generando un ectodominio α-APP soluble que es liberado al espacio extracelular (sAPPα) y un fragmento, que queda anclado a la membrana, correspondiente al extremo carboxilo terminal (APP-CTF).

Este fragmento contiene 83 aminoácidos, conocido como C83, y es procesado posteriormente por la enzima γ-secretasa que corta entre los aminoácidos 711 y 713, originando los péptidos P3 que es liberado al espacio luminal, y el AICD (dominio intracelular de la proteína precursora amiloide), que queda en el citosol.

2.4.2. Vía amiloidogénica

Esta otra vía comienza con la actuación de la enzima β-secretasa. Esta escisión tiene lugar entre los residuos de los aminoácidos Metionina671 (Met671) y Asparagina672 (Asp672) liberando al espacio extracelular un ectodominio N-terminal soluble conocido como APP soluble β (sAPPβ). Asimismo se origina un fragmento denominado C99 (99 aminoácidos) que queda anclado a la membrana y es posteriormente procesado por la enzima γ-secretasa. Esta escisión origina el Aβ, que es liberado al medio extracelular. Este péptido tiene gran potencial para unirse unos con otros y generar agregados tóxicos (Gandy, 2005).

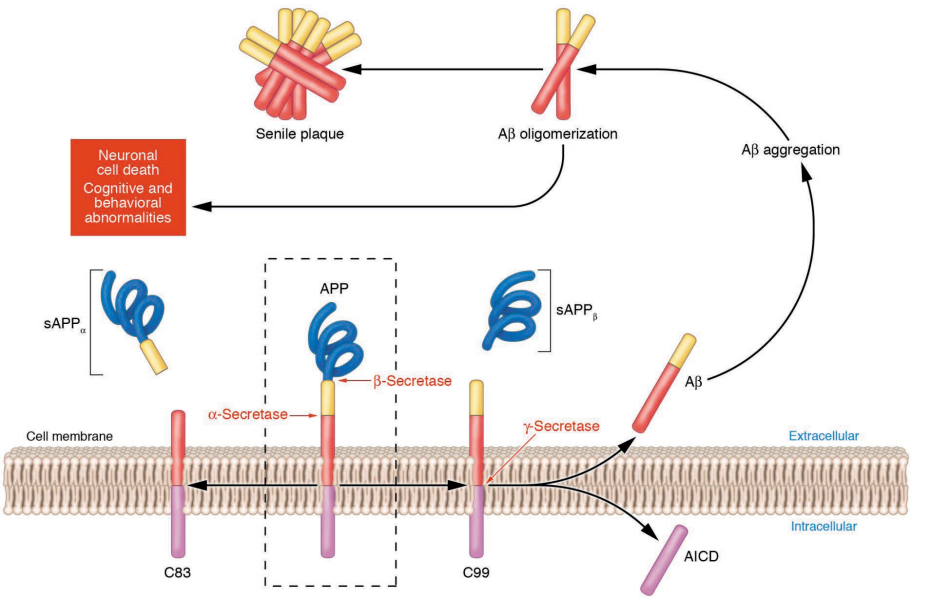


Figura 24. Procesamiento de la APP. Esquema explicativo de la ruta amiloidogénica y no amiloidogénica. A través de la ruta amiloidogénica se genera el péptido Aβ, que se acumula en el medio extracelular y forma las placas seniles características de la AD (De Strooper et al., 2010).

2.5. Enfermedad de Parkinson (PD)

La PD también denominado Parkinson idiopático o Parkinsonismo es la segunda enfermedad neurodegenerativa en frecuencia.
Afecta a más del 1 % de la población mayor de 65 años. Dos tercios de los pacientes presentan los síntomas entre los 50 y 70 años pero no es raro encontrar la enfermedad a los 40 años (Farreras & Rozman, 2012). La primera descripción de este desorden fue realizada por el médico británico James Parkinson en 1817, tras la observación de seis pacientes con los síntomas típicos de la enfermedad, a la cual denominó “parálisis agitante”. Posteriormente, fue el conocido neurólogo francés Jean-Martin Charcot quién le adjudicó el nombre de PD.

La PD es un trastorno crónico progresivo del movimiento asociado a degeneración y pérdida de neuronas dopaminérgicas en la Sustancia Negra compacta del cerebro (SNc) (Forno, 1996). La pérdida de las neuronas que se encargan de producir dopamina, neurotransmisor fundamental para el movimiento, hace que se produzcan los síntomas motores de la enfermedad.
También se ha visto la existencia de otras neuronas afectadas y por lo tanto de otros neurotransmisores como la serotonina, noradrenalina y acetilcolina, lo que conduce a la manifestación de otros síntomas no motores.

Su diagnóstico por tanto, se basa en anormalidades motoras tales como temblores en reposo, bradicinesia (lentitud en el movimiento) y rigidez e inestabilidad postural (Gibb & Lees, 1988; Dubois et al., 2010). Entre los síntomas no motores se encuentran: depresión, ansiedad y trastornos del sueño (Jenner et al., 2013). Se estima que entre el 15 y el 25 % de las personas con PD tiene algún pariente que padece la enfermedad. Las formas familiares representan entre un 5-10 % de los casos siendo el 90 % de ellos formas esporádicas.

Se ha descrito que los primeros síntomas motores de la PD aparecen tras la pérdida de aproximadamente el 60 % de neuronas dopaminégicas de la SNc (Jankovic, 2008; Wirdefeldt et al., 2011).

Microscópicamente la PD se caracteriza por la presencia de agregados proteicos amiloideos intra y extraneuronales conocidos como Cuerpos de Lewy (CL) (Figura 25). Los CL aparecen como masas esféricas que desplazan al resto de componentes celulares. El principal componente de estas estructuras es la αS, una proteína presináptica abundante en células dopaminérgicas del cerebro. Su función fisiológica permanece aún desconocida.

En su forma nativa la αS carece de una estructura secundaria definida pudiendo adoptar múltiples conformaciones en solución y en asociación con otras proteínas y membranas (Holdorff et al., 2013).

Figura 25: CL en SNc de pacientes con PD (*http://lewybody.org/sites/images/lbslewbody331x249.jpg).*

2.5.1. Etiología de la PD

La PD es una enfermedad compleja y multifactorial. Se cree que la causa de la enfermedad es una combinación de factores genéticos y medioambientales. Se ha descrito que el factor de riesgo más importante es la edad (Reeve et al., 2014), aunque existen otros riesgos asociados que se describen a continuación.

2.5.1.1. Factores genéticos

Solo el 5-10 % de los casos de la PD pueden explicarse por causas genéticas (Klein & Westenberger, 2012). Existen numerosas mutaciones relacionadas con la PD. Entre ellas 4 genes son los responsables de la mayoría de los casos: SNCA, LRRK2, PINK1, y PARK2 (Polymeropoulos et al., 1997; Di Fonzo et al., 2005; Jones, 2010).

El gen SNCA codifica la proteína αS. Las mutaciones están localizadas en el extremo N-terminal de la proteína, concretamente en las regiones: Alanina 53 Treonina (Ala53Thr), Alanina 30 Prolina (Ala30Pro) y Glutamina 46 Lisina (Glu46Lys) (Krüger et al., 1998; Spira et al., 2001; Zarranz et al., 2004).

Por otro lado el gen LRRK2 codifica para la proteína Leucine-rich Repeat Kinase2, parece ser que esta proteína está implicada en la regulación del citoesqueleto y la degradación autofágica de proteínas (Rideout & Stefanis, 2014). Han sido descrita más de 80 mutaciones de este gen, de entre ellas, 6 estarían implicadas en el desarrollo de PD: Arginina 1441 Cisteína (Arg1441Cys), Arginina 1441 Glicina (Arg1441Gly), Tirosina 1699 Cisteína (Tyr1699Cys), Glicina 2019 Serina (Gly2019Ser) e Isoleucina 2020 Treonina (Ile2020Thr) (Di Fonzo et al., 2005).

Los genes Parkin, PARK2 y PINK1 codifican dos proteínas implicadas en la degradación de las mitocondrias por los proteasomas. Por tanto, una pérdida de función de estas proteínas daría como resultado la acumulación de mitocondrias defectuosas que causarían un importante estrés oxidativo favoreciendo la neurodegeneración (Jones, 2010).

2.5.1.2. Factores ambientales

Actualmente, solo el 10 % de los casos de PD pueden ser explicados por causas genéticas. El 90 % de los casos son de origen esporádico, lo cual sugiere que esta enfermedad tiene un etiología multifactorial (Goldman, 2014).
Numerosos estudios epidemiológicos han sugerido que la exposición a tóxicos ambientales como pesticidas, metales y solventes aumenta considerablemente el riesgo de padecer la PD (Hatcher et al., 2008; Gao & Hong, 2011).

La aparición de síndrome parkinsoniano debida a la inyección intravenosa de 1-metil-4-fenil-1,2,3,6 tetrahidropiridina (MPTP) (producto derivado de la síntesis de meperidina o heroína sintética) hizo que surgiera la hipótesis sobre la relación entre la exposición de pesticidas y la aparición de la PD (Langston et al., 1983).
Este compuesto es convertido en los tejidos biológicos en 1-metil-4-fenil-2,3-dihidropiridinio (MPDP+), metabolito intermedio y finalmente en 1-metil-4-fenilpiridinio (MPP+).
El daño celular originado por este compuesto se basa en la producción intraneuronal de radicales libres citotóxicos durante la oxidación del MPP+ en cantidades tan elevadas que son imposibles de neutralizar.

El paraquat tiene una estructura muy similar al MPP+. Este plaguicida atraviesa la BHE situándose a nivel de las terminaciones nerviosas de las células dopaminérgicas promoviendo el estrés oxidativo y la muerte celular. Diversos estudios en modelos animales han puesto de manifiesto que el paraquat aumenta los ROS, reduce el número de células dopaminérgicas de la SNc y provoca la activación de microglías (células inmunitarias en el SNC) y además favorece a agregación de la αS (McCormack et al., 2002; Shimizu et al., 2003; Purisai et al., 2007).

La rotenona, otro insecticida, también atraviesa fácilmente la BHE y actúa inhibiendo el complejo I de la cadena respiratoria, hecho que provoca la generación de ROS, provocando además la activación de la microglía y favoreciendo la agregación de la proteína αS (Tanner et al., 2011).
Asimismo el insecticida organoclorado dieldrin disminuye los niveles de dopamina en el cerebro y de aumenta los ROS (Kitazawa et al., 2001).
Cabe reseñar que la utilización de estos tres pesticidas está prohibida en la Unión Europea (UE) y regulado por el Reglamento de la Comisión Europea (CE) 1107/2009.

Ciertos metales como el Hierro (Fe) y el Plomo (Pb) tienen capacidad de acumularse en el SN y generar estrés oxidativo aumentando el riesgo de desarrollar PD (Kuhn et al., 1998; Zecca et al., 2004). Por un lado, el Fe favorece la fibrilación y agregación de la αS induciendo por tanto la toxicidad en las neuronas de la SNc (Xu & Chan 2015; Li et al., 2010) y además conlleva a la formación de ROS favoreciendo la aparición de PD debido a la perturbación de su homeostasis (Sian-Hülsmann et al., 2011). La exposición a Pb y su relación con la PD ha sido bastante estudiada.
Ya en 1998, Kuhn y sus colaboradores demostraron que la exposición durante 30 años a baterías de Pb, explicaba que 7 de cada 9 trabajadores expuestos a este metal desarrollasen síntomas parkinsonianos (Kuhn et al., 1998).

A nivel molecular se ha demostrado que el Pb aumenta la peroxidación lipídica y reduce la capacidad antioxidante a nivel celular (Sandhir et al., 1994), y además promueve la fibrilación y agregación de la αS en ratas expuestas a concentraciones de entre 0-300 mg/Kg de éste metal (Yamin et al., 2003; Zhang et al., 2012).

Se han encontrado casos agudos de PD asociados a la exposición a algunos solventes como el *n*-hexano (Pezzoli et al., 1989) y el tolueno (Papageorgiou et al., 2009), entre otros.

2.5.2 Proteína αS

La αS es una proteína soluble formada por 140 aminoácidos. Esta proteína se encuentra en el citoplasma asociadas a las vesículas sinápticas por lo que se piensa que ejerce un rol en la liberación de los neurotransmisores (Cabin et al., 2002). Parece que también podría actuar inhibiendo la fusión entre las vesículas y las membranas presinápticas (DeWitt & Rhoades, 2013) aunque su

función fisiológica aún no se conoce con certeza. Como ya se ha mencionado anteriormente la αS se acumula y es el principal componente de los CL.

Esta proteína está formada por tres regiones (Figura 26):

El dominio N-terminal (residuos 1 a 60): este dominio incluye una secuencia altamente conservado: KTKEGV. Esta secuencia ante la interacción con lípidos adopta una estructura α-helicoidal (Harper et al., 1997).
El dominio central (residuos 61-95): también conocido como NAC ('Non-Amyloid-Component' o Componente No Amiloideo). También se localizan dos motivos KTKEGV en este dominio.
La región NAC es la que contiene la mayor cantidad de aminoácidos hidrofóbicos y debido a esto se la asocia con la agregación de proteínas (Iwai et al., 1995).

La región C-terminal (residuos 96-140): contiene una gran proporción de residuos ácidos y prolina y tres residuos tirosina, que se consideran una forma de la familia de α y β sinucleínas.
La presencia de aminoácidos cargados negativamente y prolinas sugiere que esta región tiene una tendencia a permanecer en una conformación desordenada, es decir no forma hojas β plegada o α hélice.Por tanto, a este dominio se le asocia un efecto "protector" contra la agregación, la interactuación con la parte NAC impide cambios en la estructura de esta región de la proteína manteniendo por consiguiente una conformación estable que evita la oligomerización y la formación de fibrillas (Hong et al., 2011).

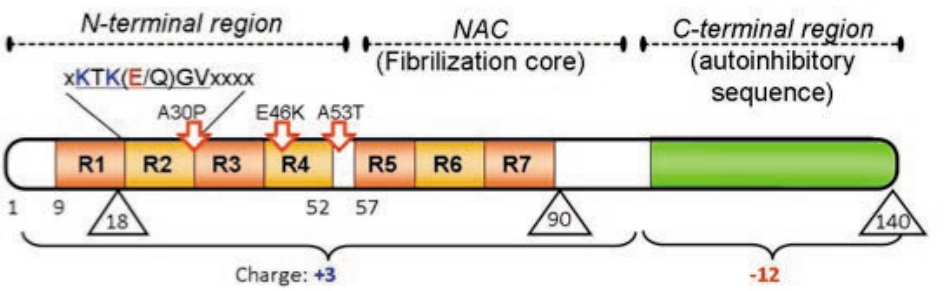


Figura 26: Regiones de la proteína αS.
(*http://www3.mpibpc.mpg.de/groups/jovin/uploads/ResearchSubjects/ASNseq.jpg*)

2.6. Agregación del péptido Aβ y de la proteína αS

Los procesos de agregación proteica en la AD y en la PD se caracterizan por ser lentos y organizados. Uno de los modelos más aceptados para la formación de estructuras amiloidogénicas es el de nucleación-polimerización (Jarret et al., 1993; Harper & Lansbury, 1997; Soto, 2001). Este modelo está caracterizado por una cinética particular definida por dos fases de agregación:

(i) fase de nucleación o fase lag, en la cual los monómeros sufren cambios conformacionales y se asocian entre ellos para formar un núcleo.

(ii) fase de elongación o fase de crecimiento, en la cual el núcleo crece debido a la adición de más monómeros que van originando largos polímeros y fibras hasta la saturación.

La fase de nucleación es termodinámicamente desfavorable y ocurre gradualmente mientras que la fase de elongación es un proceso mucho más favorable y ocurre más rápidamente. Así, este proceso puede representarse en forma de curva sigmoidal (Figura 27).

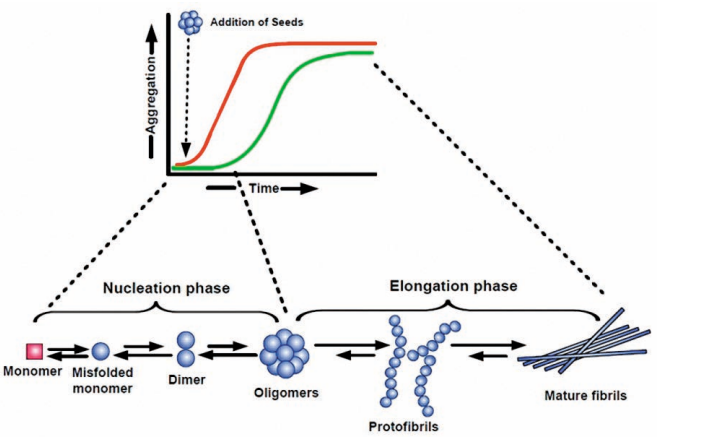


Figura 27: Proceso de agregación de proteínas amiloideas según el modelo de nucleación-polimerización (Kumar & Walter, 2011).

En condiciones fisiológicas, los monómeros de Aβ son solubles, pero en condiciones patológicas su conformación cambia a la conformación de hoja β plegada (Kelly, 1998).
Los monómeros se pliegan incorrectamente; se autoagregan y dan origen a agregados tóxicos (Dahlgren et al., 2002). En el proceso patológico de la agregación de Aβ pueden encontrarse intermediarios neurotóxicos como oligómeros no fibrilares pequeños y solubles denominados protofibrillas (Kayed et al., 2003). Los agregados tóxicos continúan uniéndose dando lugar a fibras y posteriormente a placas amiloides, las cuales pueden ser observadas en el tejido cerebral de pacientes que padecen AD (Pike et al., 1993).

En el caso de la αS el monómero de la proteína se encuentra en forma no estructurada. La parte N-terminal adquiere una conformación en forma α-hélice, hecho que parece favorecer la interacción de la proteína con la membrana presináptica.
La agregación de monómeros no estructurados de αS lleva a la formación de oligómeros lo cual es posible ya que estos monómeros se encuentran con la región NAC expuesta permitiendo que las diferentes proteínas de αS interactúen y se vayan estructurando en forma de hoja β plegada.

En este proceso de agregación pueden formarse dos tipos de agregados: por un lado oligómeros (polímeros estables de αS no estructurada) y las protofibrillas (agregados estructurados de αS que formarán fibrillas tras sufrir el proceso de elongación) (Conway et al., 2000). Las protofibrillas están formadas por la unión de entre unas 20-30 proteínas aproximadamente (Lashuel et al., 2002). Finalmente, estos agregados se estructuran en fibrillas que darán lugar a los CL.

Actualmente se admite que las protofibrillas son los agregados más tóxicos generados en el proceso de agregación (Volles & Lansbury, 2002; Winner et al., 2011; Lashuel et al., 2013). Estos agregados dan lugar a la neurodegeneración característica en ambas enfermedades. Diversos mecanismos fisiopatológicos han sido descritos como responsables de la neurotoxicidad:

- Disfunción proteosomal
- Disminución de Glutatión (GSH)
- Disfunción mitocondrial
- Daño en el ADN
- Oxidación de proteínas y peroxidación lipídica
- Producción de ROS
- Neuroinflamación y proliferación de células gliales

Este mecanismo subyacente en diversos procesos neurodegenerativos es la base para el diseño de diversas estrategias de prevención y tratamiento de estas enfermedades.
Entre ellos podemos destacar la búsqueda de compuestos que puedan interferir en la agregación de las proteínas o en la desestabilización de las fibras formadas.
Para comprobar esta hipótesis se utilizan diversas técnicas *in vitro* para:

- Realizar un seguimiento de la cinética de agregación y comprobar si los compuestos bloquean este proceso.
- Comprobar las formas resultantes de las proteínas al ser tratadas con estos compuestos.
- Probar los efectos antioxidantes y neuroprotectores de los compuestos frente a la toxicidad del péptido Aβ y de la proteína αS.

2.7. Efectos de los compuestos polifenólicos, melatonina y otros indoles en la AD y la PD

En este apartado se resumen los antecedentes del efecto de bioactivos presentes en los alimentos frente a la formación de fibras de Aβ y αS y en la desestabilización de fibras ya formadas, así como ensayos para comprobar la actividad frente a su toxicidad con modelos celulares *in vitro* o *in vivo* en modelos animales.

Uno de los compuestos más estudiados ha sido la curcumina, polifenol presente en la cúrcuma (*Curcuma longa*) la cual es capaz de inhibir la agregación del Aβ y desestabilizar fibras además de atenuar la citotoxicidad producida por Aβ *in vitro* (Ono et al., 2004). Tras la administración oral de 500 mg/Kg de curcumina, se han encontrado unas concentraciones en plasma de 0,16±0,03 mM (Yang et al., 2007). Además se ha comprobado que este compuesto puede atravesar la BHE en ratones Tg2576 (ratones transgénicos que sobreexpresan la APP) (Yang et al., 2004).

Otro de los compuestos más estudiados ha sido la epigalocatequina-3-galato (EGCG), bioactivo presente en el té verde. Diversos estudios han demostrado que la EGCG inhibe la agregación de Aβ; este compuesto es capaz de unirse con la proteína en su estado nativo y evitar la formación de oligómeros tóxicos (Bieschke et al., 2010). Diferentes estudios de biodisponibilidad indican un valor de 1,52±1,36 mg/mL (tras el consumo de 800 mg de EGCG en voluntarios humanos sanos) (Chow et al., 2005).
Por otro lado, diversos estudios en modelos animales han confirmado que este compuesto es capaz de atravesar la BHE llegando a una concentración de 0,5 nmol/g en cerebro tras ingerir 500 mg/Kg (Adachi et al., 2006).

Otros polifenoles también se han relacionado con la protección frente a AD. Entre estos se incluyen: miricetina, morina, quercetina, kaempferol, (+)-catequina y (-)-epicatequina. Se ha demostrado que estos compuestos inhiben la agregación del $A\beta_{1\text{-}40}$ y del $A\beta_{1\text{-}42}$ de manera dosis dependiente (Ono et al., 2003).

El resveratrol, estilbeno presente en el vino tinto, ha demostrado capacidad de retrasar la progresión de trastornos neurodegenerativos (Ramassamy, 2006). Feng et al. (2009) demostraron que este compuesto inhibe tanto la extensión como la polimerización de Aβ además de atenuar la toxicidad inducida por este péptido en células de neuroblastoma (SY-SY5Y). Similares hallazgos se han publicado en neuronas primarias del hipocampo obteniéndose un efecto dosis-dependiente (Han et al., 2004).
Además en células neuronales (PC12) se ha comprobado que el resveratrol es capaz de proteger frente a la toxicidad del Aβ mediante cambios en las vías de señalización de la apoptosis, reduciendo los cambios en el potencial de la membrana mitocondrial e inhibiendo la acumulación intracelular de ROS (Jang & Surh, 2003). En cuanto a la biodisponibilidad del resveratrol, se han

encontrado en plasma concentraciones de sus metabolitos de hasta 491 ng/mL (alrededor de 2 mM), sin embargo, se encuentran menos de 5 ng/mL de resveratrol en su forma original tras la administración oral de 25 mg en humanos (Walle et al., 2004). Además de este compuesto, otros monómeros y dímeros de estilbenos como el piceido, el escipursin A o la ε-viniferina glucósido han demostrado poseer un efecto muy similar o incluso mayor que el resveratrol en la inhibición de la agregación de Aβ *in vitro* (Rivière et al., 2010).

De la misma forma se ha demostrado la actividad de varios polifenoles frente a la agregación y desestabilización de la proteína αS. Así, la curcumina también es capaz de inhibir la agregación y aumentar la solubilidad de la αS en células que contienen agregados de manera dosis-dependiente (Pandey et al., 2008).
Otros polifenoles también han demostrado actividad antiinhibitoria y desestabilizadora de fibras de αS: EGCG (Ehrnhoefer et al., 2008; Bieschke et al., 2010), ácido rosmarínico (Rao et al., 2008), ácido tánico, miricetina, kaempferol, (+)-catequina y (-)-epicatequina (Ono & Yamada et al., 2006) y ácido gálico (Ardah et al., 2014).

Se ha observado un descenso de los niveles de melatonina y de sus precursores (triptófano y serotonina) en personas con edad avanzada, hecho que se ha asociado con la aparición de AD (Zhou et al., 2003; Greilberger et al., 2010).

Tal y como se describió anteriormente, la fresa presenta otros bioactivos tales como la melatonina y de los que existe evidencia de sus efectos neuroprotectores. Se ha comprobado que esta neurohormona puede interactuar con protofibrillas de Aβ e prevenir la producción de ROS, mantener los niveles intracelulares de calcio así como los niveles de acetilcolina en ensayos *in vivo* (Masilamoni et al., 2008).
Se ha comprobado que la melatonina protege a las neuronas y células gliales de la muerte provocada por $A\beta_{1–40}$, $A\beta_{25–40}$ y $A\beta_{1–28}$ incrementando la viabilidad celular *in vitro*.
Este mismo efecto también se ha demostrado en células de neuroblatoma N2a y células PC12 tras la exposición a los efectos tóxicos del $A\beta_{25–35}$ (Pappolla et al., 1997; Ionov et al., 2011).

Skribanek et al. (2001) comprobaron una interacción entre la melatonina y el Aβ, específicamente entre los residuos 20-40 de esta proteína. Además se le han atribuido efectos inhibidores en la formación de estructuras de hojas β, gracias a interacciones electrostáticas en los residuos de los aminoácidos histidina y aspártico que promueven la disolución de las fibras (Fraser et al., 1991; Huang et al., 1997; Pappolla et al., 1998).

En el caso de los efectos neuroprotectores frente a la AD relacionados con otros índoles derivados del metabolismo del triptófano no existe demasiada bibliografía, siendo este un campo de interés actual. Morshedi et al. (2007) probaron los efectos inhibitorios de varios derivados indólicos frente a la agregación de la proteína lisozima, modelo utilizado debido a que también posee

propiedades de agregación tipo amiloidogénico.
El ácido 3 indolácetico y el triptofol fueron los dos compuestos que mostraron bioactividad en este estudio.

Con respecto al papel de la melatonina en PD diferentes estudios han demostrado un efecto positivo explicado por diferentes mecanismos de acción. Así, Lin et al. (2007) demostraron que la melatonina presenta capacidad de reducir los niveles de agregados de αS en cerebros de rata mediante técnicas de western blot, atenuando los efectos apoptóticos de la arsenita. Además se ha demostrado que la melatonina inhibe la formación de fibras de αS en células de feocromocitoma de rata (Ishido, 2007).

Ono et al. (2012) observaron una reducción en el número de fibras con el consiguiente incremento de fibras más cortas y agregados con formas amorfas tras la incubación con melatonina. Además la melatonina posee un efecto desestabilizador de las fibras ya formadas.
En este mismo estudio se comprobó en células primarias que este compuesto reduce los efectos tóxicos de la αS en sus diferentes formas (2 y 6 días de incubación) observándose un incremento significativo de la viabilidad celular de entre un 56-97 %.

Mayo et al. (1998) comprobaron que esta hormona es un efectivo capturador de ROS y previene la apoptosis en células neuronales. Además otros ensayos en ratón han demostrado que la melatonina protege frente a la toxicidad del MPTP ya que reduce la autooxidación de la dopamina (Escames et al., 2010).

Estos antecedentes muestran la oportunidad de estudio del efecto de otros compuestos indólicos potencialmente presentes en alimentos frente a la agregación de estas proteínas los cuales han sido poco estudiados. La revisión de estos efectos se muestra en el Anexo I.

Justificación y Objetivos

La fresa es un fruto de carácter estacional y perecedero lo cual hace que se produzcan excedentes de su producción haciendo que surja la necesidad de elaborar productos derivados, tales como bebidas fermentadas, con el objetivo de alargar su vida útil, evitar pérdidas económicas y ofrecer productos innovadores para el consumidor.

Es un fruto caracterizado por ser una fuente rica en vitaminas antioxidantes y bioactivos entre los que destacan los compuestos polifenólicos, a los cuales han sido atribuidas numerosos efectos saludables.

Los procesos biotecnológicos a los que se somete este fruto dan lugar a modificaciones significativas de los compuestos fenólicos, entre ellos la fracción antociánica, responsable del color. Por ello, resultan de gran interés la caracterización química de estos nuevos alimentos, el estudio los factores que afectan a su estabilidad y la evaluación de su bioactividad.

La melatonina ha sido recientemente descrita en fresa y en otros alimentos fermentados. Es una neurohormona que se utiliza como suplemento dietético y que posee propiedades beneficiosas para la salud entre los que destacan la regulación del sueño, actividad antioxidante y antitumoral.

La búsqueda de compuestos presentes en los alimentos que componen la dieta y su papel en la neuroprotección ha suscitado muchas expectativas como estrategias para la prevención de enfermedades neurodegenerativas, tales como el Alzheimer y el Parkinson, que suponen un gran problema sanitario debido a la creciente incidencia, a la falta de un tratamiento eficaz que contrarreste lo síntomas y a la inexistencia de una cura definitiva.

La presente Tesis Doctoral tiene como objetivo general la evaluación de bebidas obtenidas a partir de distintas fermentaciones de fresa.

Para ello se han definido los siguientes objetivos específicos:

a) Caracterización química del perfil antociánico de fermentados alcohólicos, acéticos y glucónicos elaborados a partir de puré de fresa.

b) Evaluación de la actividad antioxidante y color de fermentados de fresa.

c) Estabilidad de la fracción antociánica, color y actividad antioxidante de una bebida elaborada a partir de fermentados de fresa.

d) Efectos neuroprotectores de fermentados alcohólicos y glucónicos de fresa frente a la neurotoxicidad del péptido Aβ y a la producción de ROS.

e) Evaluación de la actividad del ácido protocatéquico (metabolito de antocianos) frente a la agregación y toxicidad del péptido Aβ y de la proteína αS.

f) Evaluación de la bioactividad de la melatonina y compuestos relacionados frente a la agregación y toxicidad del péptido Aβ.

Materiales

En la realización de la presente Tesis Doctoral se han empleado los siguientes materiales:

Muestras fermentadas de fresa

En este trabajo se han evaluado distintos productos fermentados elaborados a partir de puré fresa.

El puré de fresa, sustrato de la fermentación, procedente de las campañas 2012 y 2013, fue suministrado por la compañía Hudisa Desarrollo Industrial S.A. (Lepe, España).
Este puré fue elaborado a partir de excedentes de las siguientes variedades de fresa: *Camarosa, Candonga, Festival, Ventana, Splendor, Honor* y *Coral* aunque mayoritariamente es *Camarosa* (>90%). Una vez la fresa es recibida en las instalaciones de la empresa, se selecciona y limpia y se procede a la eliminación de las hojas y tallos.
Seguidamente se tritura y se lleva a cabo un proceso de calentamiento a 55-65 ºC durante 2 minutos con el objetivo de inactivar las enzimas presentes.
A continuación, se pasteuriza a 90 ºC durante 3 minutos, reduciéndose la temperatura a 5 ºC una vez finalizado este proceso. Finalmente se realiza un tamizado para eliminar las semillas y una posterior congelación a 20 ºC.

Las fermentaciones se han llevado a cabo en el Departamento de Química Inorgánica e Ingeniería Química de la Universidad de Córdoba (España) a escala de laboratorio a partir del puré de fresa descrito anteriormente dentro del marco del Proyecto del Plan Nacional "Evaluación de la Calidad y Seguridad de una Nueva Bebida Obtenida a partir de Fresa no apta para Comercialización" y en concreto el subproyecto 3: "Procesos de Fermentación para la Producción de una Nueva Bebida a partir de fresa no apta para Comercialización" (Investigador Principal: Dr. Isidoro García).
Los inóculos fueron proporcionados por el grupo de la Universidad Rovira i Virgili, encargados del subproyecto 2: "Selección y Control Microbiológico para la producción de una Nueva Bebida a partir de fresa no apta para Comercialización" (Investigadora Principal: Dra. Mª Jesús Torija).

Las fermentaciones acéticas, alcohólicas y glucónicas se realizaron en cultivo sumergido.
Este sistema se basa en la presencia de un cultivo de microorganismos sumergidos libremente en el líquido a fermentar que se distribuye de forma homogénea con ayuda de agitación mecánica. El suministro de oxígeno en el fermentador se realiza de manera automática en función de las variables operacionales programadas en cada experimento. Se fija como valor constante el porcentaje de oxígeno disuelto, así cuando este nivel es inferior al programado el sistema airea automáticamente restableciendo estos valores.
Cabe destacar que la fermentación acética y glucónica son procesos aerobios mientras que la alcohólica es un proceso anaerobio.

A continuación se detallan las condiciones de fermentación de los distintos fermentados.

Fermentación Alcohólica

Se utilizó la levadura ***Saccharomyces cerevisiae*** (CECT 13057) aisladas de la propia fresa (Hidalgo et al., 2013). Se realizó una fermentación en modo semicontinuo a partir de un volumen inicial de 3,6 L de puré de fresa.

Las condiciones utilizadas fueron: temperatura de 29 ºC y agitación a 250 rpm.
El medio se saturó con oxígeno al principio de la fermentación antes de que el inóculo fuese añadido. Se utilizó un inóculo (125 mL) con la siguiente composición: 10 % (p/v) glucosa, 0.1 % (p/v) $MgSO_4$, 0.2 % (p/v) KH_2PO_4, 0.3 % (p/v) $(NH4)_2SO_4$, 0.4 % (p/v) extracto de levadura y 0.36 % (p/v) de peptona bacteriológica. La fermentación finalizó una vez los azúcares fueron consumidos y el pH alcanzó un valor de 3.30.

Fermentación acética

Para la preparación del inóculo se utilizó un cultivo mixto de bacterias acéticas autoseleccionadas, concretamente de los géneros ***Acetobacter y Gluconobacter,*** procedente de un acetator Frings (Baena-Ruano et al., 2010).
La fermentación se realizó en modo semicontinuo y las condiciones fueron: temperatura de 31 ºC, agitación a 500 rpm y oxígeno disuelto al 70 %.
Al inicio, el acetificador se cargó con 3.6 L de fermentado alcohólico de fresa, y una vez el alcohol fue consumido, se descargaron 2.6 L y se reemplazaron por este mismo volumen con fermentado alcohólico de fresa.

Fermentación glucónica

En este caso se utilizaron bacterias del género ***Gluconobacter japonicus*** (CECT 8443) para el proceso fermentativo la cual fue aislada originalmente de mosto de uva procedente de la bodega experimental del grupo del Dr. Albert Mas (Mas dels Frares, Tarragona) (Navarro, 2011).
Como sustrato se utilizó puré de fresa (3 L), que fue introducido en el fermentador y se trabajó en modo discontinuo (batch) con las siguientes condiciones: pH 3.24, temperatura de 29 ºC y 500 rpm.
Se añadieron 125 mL de inóculo de *G. japonicus* con la siguiente composición: 5 % (p/v) glucosa, 1 % (p/v) extracto de bacteria y 2 % (p/v) peptona bacteriológica, y se mezcló durante 20-30 minutos. El final de la fermentación se estableció en el momento en el que la glucosa fue totalmente consumida y el valor de pH 2.74.

Todas las muestras fueron sometidas a un tratamiento previo para la extracción de la fracción antociánica cuyo procedimiento se detalla a continuación:

Para la extracción de la fracción antociánica se utiliza una resina específica, la amberlita (XAD7HP), la cual es capaz de adsorber y liberar especies iónicas gracias a interacciones polares e hidrofóbicas. Esta resina fue activada con metanol y empaquetada en una columna de vidrio (30 x 1.5 cm). Se tomaron 20 g de cada una de las muestras y se diluyeron con agua destilada (1:1 p/v). La muestra fue introducida en la columna y se repitieron varios ciclos de limpieza. Finalmente la fracción antociánica se eluyó con una mezcla de metanol/ácido acético (19:1) con un flujo de una gota/minuto. La fracción recuperada fue concentrada en un rotavapor a vacío. Finalmente el extracto se reconstituyó en 2 mL con agua acidificada (5 % ácido fórmico) y se congeló a -20 ºC hasta su análisis.

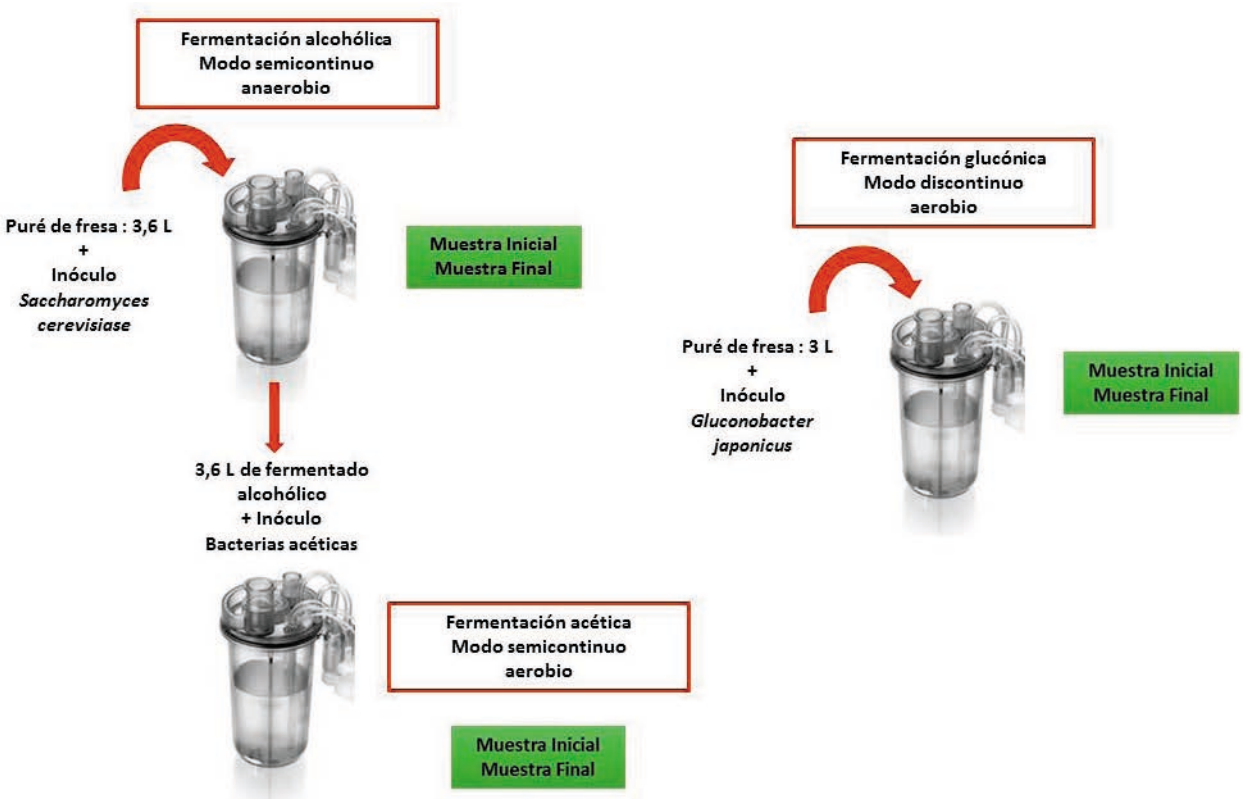
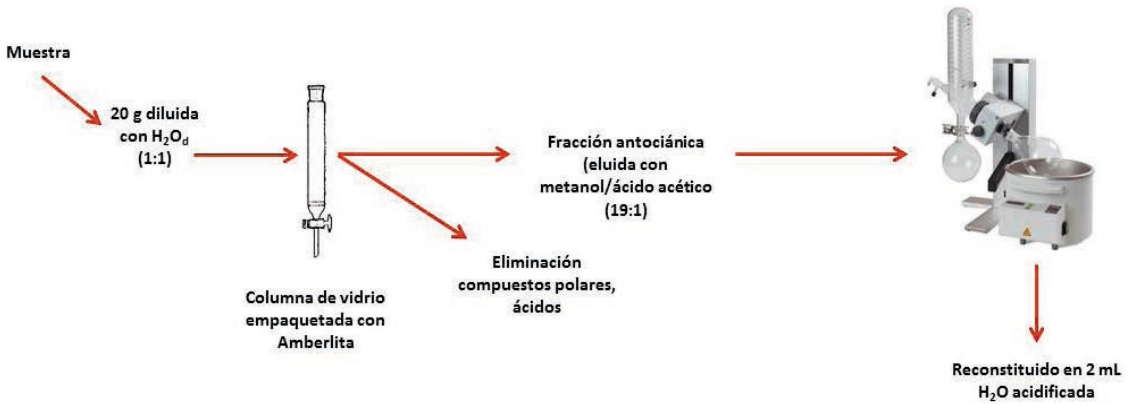


Figura 28:
Fermentaciones a partir de puré de fresa como sustrato y muestras resultantes evaluadas en esta Tesis Doctoral.

Figura 29:
Proceso de extracción de la fracción antociánica.



Por otro lado se ha elaborado y evaluado una bebida a partir de fermentados de fresa cuya composición fue:

A: Fermentado glucónico de fresa centrifugado y diluido con agua carbonatada (80/20)

B: Vinagre de fresa

Bebida: 99,04 % A + 0,96 % B (4 gotas de Stevia/100 mL (Marca comercial: Ecosana (60% de Rebaudiósido A))

Para el análisis de su estabilidad se almacenaron a temperatura ambiente (25 ºC) y temperatura de refrigeración (4 ºC) a distintos tiempos: 0, 15, 30, 60 y 90 días.

	Nomenclatura de las muestras		Ciclos y número de muestras
Capítulo 1	Inicial		3 muestras (duplicado)
	Final		
	•	Vino de fresa	3 ciclos de fermentación (duplicado)
	•	Vinagre de fresa	2 ciclos de fermentación (duplicado)
			n=16
Capítulo 2	A12I	Alcohólica Inicial Campaña 2012	
	A12F	Alcohólica Final Campaña 2012	8 ciclos de fermentación alcohólica
	A12FP	Alcohólica Final Pasteurizada Campaña 2012	(4 con puré de fresa campaña 2012
	A13I	Alcohólica Inicial Campaña 2013	y 4 con puré de fresa campaña
	A13F	Alcohólica Final Campaña 2013	2013; duplicado)
	A13FP	Alcohólica Final Pasteurizada Campaña 2013	*n=24*
	G12I	Glucónica Inicial Campaña 2012	
	G12F	Glucónica Final Campaña 2012	6 ciclos de fermentación glucónica
	G12FP	Glucónica Final Pasteurizada Campaña 2012	(4 con puré de fresa campaña 2012
	G13I	Glucónica Inicial Campaña 2013	y 2 con puré de fresa campaña
	G13F	Glucónica Final Campaña 2013	2013; duplicado)
	G13FP	Glucónica Final Pasteurizada Campaña 2013	*n=18*
Capítulo 3	Inicial (0)		
	R15: bebida almacenada a 25 ºC durante 15 días		
	R30: bebida almacenada a 25 ºC durante 30 días		
	R60: bebida almacenada a 25 ºC durante 60 días		Todas las muestras analizadas por duplicado
	R90: bebida almacenada a 25 ºC durante 90 días		
	F15: bebida almacenada a 4 ºC durante 15 días		*n=18*
	F30: bebida almacenada a 4 ºC durante 30 días		
	F60: bebida almacenada a 4 ºC durante 60 días		
	F90: bebida almacenada a 4 ºC durante 90 días		

Tabla 5:
Muestras de fermentados y bebida de fresa.
Muestras iniciales (puré de fresa + inóculo antes de comenzar la fermentación; I). Muestras finales (final de la fermentación; F). Muestras finales pasteurizadas (muestra final pasteurizada a Tª entre 70-80 ºC durante 15 minutos; FP).

Reactivos

- Folin-Ciocalteu; acetato de sodio (Merk)
- KCl; Na_2HPO_4; NaH_2PO_4; NaCl (Panreac)
- Ácido gálico; fluoresceína sodio; ácido 3-indolacético; acetato de uranilo (Fluka)
- Amberlita XAD7HP; dimetilsulfóxido (DMSO); medio de cultivo Dulbecco´s modified Eagle´s (DMEM)-Glutamax; tripsina-EDTA, bromuro de 3-(4,5- dimetiltiazol-2-ilo)-2,5-difeniltetrazol (MTT); tampón fosfato salino (PBS), L-glutamina, suero de caballo fetal; suero bovino fetal; estreptomicina; diclorofluoresceína diacetato (DCFH-DA); 2,2-difenil-1-picrilhidrazilo (DPPH); 2,2 '-azo-bis (2-amidino-propano) dihidrocloruro (AAPH); Trolox (ácido 6-hidroxi-2,5,7,8-tetrametilcroman-2-carboxílico; ácido protocatéquico; tioflavina T (ThT); triptamina; serotonina; triptofol; n-acetil-5-hidroxitriptamina; n-acetilserotonina; melatonina; Tween 20 (Sigma Aldrich)
- Metanol; ácido acético (VWR Chemicals)
- Ácido fórmico; acetonitrilo (Fisher Chemical)
- Pelargonidina 3-glucósido; cianidina 3-glucósido; delfinidina 3-glucósido; peonidina 3-glucósido (Chromadex Inc)
- Celúlas PC12-Adh (ATCC® CRL-1721.1TM)
- Péptido β-amiloide fragmento 25–35 ($A\beta_{25-35}$) (Synvec)
- Péptido $A\beta_{1-42}$; proteína αS (Alexotech)
- Anticuerpo: anti-human amyloid-B (N) (82E1) mouse IgG MoAb (IBL)
- Anticuerpo monoclonal: beta amyloid, 1-16 (6E10) (Covance)
- Anticuerpo: anti-mouse IgG, HRP-linked (Cell Signaling Technology)
- Marcador de proteínas: HyperPAGE Prestained Protein Marker (Bioline)
- Solución 10 % SDS (v/v); glicina; azul de bromofenol; glicerol anhidro (Applichem)
- 2-mercaptoetanol; 10x Tris/Glicina/SDS (10x tampón de electrophoresis compuesto por: 25 mM tris, 192 mM glicina, 0,1 % SDS pH 8,3); 10x Tris/Glicina (10x tampón de electroforesis, pH 8,3); gel de poliacrilamida 4-20 % Mini-PROTEAN® TGX; membrana Immune-Blot PVDF; azul de Coomassie (Bio-Rad)
- Revelador de quimioluminiscencia: Pierce, ECL 2 Plus Western Blotting Substrate (Thermo Scientific)
- Rejillas recubiertas de carbono para microscopía electrónica: Carbon-coated grids, 300mesh, copper (EMS).

Instrumentación

- Espectrofotómetro (U-2800,Hitachi)
- Espectrofotómetro (Konica Minolta, CM-3600d, Minolta Co. Ltd.).
- UHPLC acoplado a espectrómetro de masas híbrido quadrupolo-orbitral (Q Exactive) de alta resolución con fuentes de ionización ESI, APCI y nanoESI (HESI-II, Thermo Fisher Scientific).
- UHPLC acoplado a detector de diodos (DAD) y a espectrómetro de masas con fuentes de ionización ESI, APCI y trampa de iones como analizador (1290 Infinity, Agilent).
- Espectrofluorímetro (FLUOstar Optima, BMG Labtech).
- Lector de microplacas (MRXII, Dynex).
- Lector de microplacas (Synergy HT, Biotek).
- Microscopio electrónico de transmisión (Libra 120, Zeiss).
- Equipo de quimioluminiscencia (Amersham Imager 600, GE Healthcare Life Sciences).
- Equipo de PCR convencional TC-5000 (Techne).
- Cubeta de electroforesis Mini-PROTEAN Tetra Cell (Bio-Rad).
- Cabina de seguridad biológica (BIO II A, TELSTAR).
- Incubador de CO_2 (NU-4750-E US, NUAIRE).
- Centrifuga multitarea refrigerada (Allegra X-22R, Beckman Coulter).
- Microscopio invertido de contraste de fase (CKX41SF2, Olympus).
- Baño termostatizado (BAE-2, RAYPA).

Capítulo 1

Influencia de distintos procesos de fermentación en la composición antociánica de bebidas elaboradas a partir de fresa

Influence of different fermentation processes on the anthocyanin composition of beverages elaborated from strawberry

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Influence of fermentation process on the anthocyanin composition of wine and vinegar elaborated from strawberry

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Keywords:	anthocyanin, fermentation, wine, vinegar, strawberry

Section: Food Chemistry

Title: Influence of fermentation process on the anthocyanin composition of wine and vinegar elaborated from strawberry

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Short version of title: Anthocyanins on strawberry wine & vinegar

Abstract

Anthocyanins are the major polyphenolic compounds in strawberry fruit responsible for its color. Due to their reactivity, they are affected by food processing techniques as fermentation that alters both their chemical composition and organoleptic properties. This work aims to evaluate the impact of different fermentation processes on individual anthocyanins compounds in strawberry wine and vinegar by (UHPLC-MS/MS) Q Exactive analysis. Nineteen, eighteen and fourteen anthocyanin compounds were identified in the strawberry initial substrate, strawberry wine and strawberry vinegar, respectively. Four and eight anthocyanin compounds were tentatively identified with high accuracy for the first time to be present in the beverages obtained by alcoholic fermentation and acetic fermentation of strawberry respectively. Both the total and the individual anthocyanin concentrations were influenced by both fermentation processes, affecting the alcoholic fermentation in a lesser extent than the acetic fermentation. Indeed, several changes in color parameters have been assessed. The color of the wine and the vinegar made from strawberry changed during the fermentation process, varying from red to orange color, this fact is directly correlated with the decreased of anthocyanins compounds.

Keywords: Anthocyanins, fermentation processes, strawberry, wine, vinegar

Introduction

Strawberries and derived strawberry products are considered a rich source of phytochemicals (ellagic acid, anthocyanins, quercetin and catechin), vitamins C and E and folic acid (Proteggente and others 2002; Stürtz and others 2011). Although this fruit is widely consumed as fresh product, currently there are more and more processed products commercially available (spreads, jams, syrups, alcoholic and nonalcoholic beverages, teas, and so forth). Furthermore, innovative trends in designing food products are devoted to satisfy consumer's demand of a higher diversity on the market. In addition to the fact that the transformation of a high perishable fruit into beverages permits to increase its conservation period and prevent food waste, fermentation has been proposed to increase the bioaccesibility of the polyphenolic compounds by their release from the vegetal matrix (Acosta-Estrada and others 2014).

It is color that represents an aspect of major importance on strawberry quality. Indeed, attractive colors increase consumer's preference and consequently, its price. Not only, does this parameter that influence on the quality of the beverage but also on its chemical composition. In this context, anthocyanins compounds are relevant due to their contribution on color and bioactivity (Basu and others 2014). Furthermore, it is well know that pelargonidin 3-glucoside is the major anthocyanin in strawberry (153–652 mg/kg fresh weight) followed by pelargonidin 3-rutinoside and other pelargonidin and cyanidin derivatives (Lopes-da-Silva and others 2007; Cerezo and others 2010).

Anthocyanins are known to be unstable compounds. Food processing, time, storage and temperature are crucial factors that influence significantly the stability of these

66 compounds (Clifford 2000). These factors can lead to several chemical and enzymatic
67 reactions that produce a change or modification of their chemical structure (Cavalcanti
68 and others 2011). In fact, changes in anthocyanin compounds during wine–making
69 process and vinegar elaboration have been studied in depth. For example,
70 pyranoanthocyanins constitute one of the most important classes of anthocyanin-
71 derived pigments occurring naturally in red wine (de Freitas and Mateus 2011; Oliveira
72 and others 2010). Moreover, it have been established that anthocyanins with acylating
73 substituents are more stable during processing and storage (Fossen and others 2010;
74 Giusti and Wrolstad 2003). This fact has been attributed to the stacking of the acyl
75 groups with the pyrilium ring of the flavylium cation, reducing the susceptibility of a
76 nucleophile attack of water and therefore the formation of intramolecular
77 copigmentation of a pseudobase or a chalcone (Brouillard and others 2003). Despite
78 that the characterization of anthocyanin composition of strawberry has been
79 accomplished lately, studies devoted to their profile in beverages made from
80 strawberry are rather scarce (Hornedo-Ortega and others 2016 a, b). Most of the
81 studies of anthocyanins composition in strawberry derived products have been
82 conducted to determine their total anthocyanin content expressed as a chemical
83 index. For example, Ubeda (2013) reported a decrease in total anthocyanins after
84 alcoholic and acetic fermentation process of strawberry. Klopotek (2005) showed a
85 loss of total anthocyanins when comparing the strawberry mash with the strawberry
86 wine.

87 However, very limited information about the individual anthocyanin composition of
88 wines and vinegars made from strawberry is available. Consequently, it is relevant to
89 gain knowledge on anthocyanin characterization to understand the effect of

fermentation on this class of bioactive compounds which in turn exert a role on sensory properties, color, in particular.

The aim of this paper is to characterize the individual anthocyanins composition of strawberry derived products obtained by alcoholic and acetic fermentation processes by means of (UHPLC-MS/MS)-Q Exactive and to evaluate the influence of these processes on the color of the resulting products.

Material and methods

Reagents and standards

Folin-Ciocalteu reagent and sodium acetate were purchased from Merk (Darmstadt, Germany); potassium chloride was supplied by Panreac (Castellar del Vallès, Barcelona); gallic acid was purchased from Fluka (Steinheim, Germany); Amberlite XAD7HP Sigma, (Steinheim, Germany); methanol, acetic acid, formic acid and acetonitrile were obtained by VWR Chemicals, (Llinars del Vallés, Barcelona); pelargonidin 3-glucoside, cyanidin 3-glucoside, delphinidin 3-glucosise and peonidin 3-glucoside were purchased from Chromadex Inc. (Irvine, California, USA).

Raw material

Strawberry purée elaborated by Hudisa, S.A. (Lepe, Spain) was used as substrate. The purée (2012 harvest) was industrially pasteurized at 92°C for 90-120 s and stored at 0-4 °C, containing 34.00 ± 1.25 g sugars/L (45% glucose and 55% fructose).

Fermentation processes

Fermentation runs were conducted in a 5 L Biostat® fermentation tank equipped with pH, agitation, dissolved oxygen and temperature controls.

Alcoholic fermentation

A *Saccharomyces cerevisiae* strain (CECT 13057), isolated from native strawberry yeast (Hidalgo and others 2013) was used as a starter for the alcoholic fermentation process. The fermentation was operated in a batch mode, using a loading volume of 3.6 L of strawberry purée. The operational conditions were: temperature, 29 °C; and agitation, 250 rpm. The medium was saturated with oxygen only at the beginning of the fermentation process before the inoculum was added [10% (w/v) glucose, 0.1% (w/v) $MgSO_4$, 0.2% (w/v) KH_2PO_4, 0.3% (w/v) $(NH_4)_2SO_4$, 0.4% (w/v) yeast extract and 0.36% (w/v) bacteriological peptone]. The alcoholic fermentation process was stopped once sugars were depleted. In total, three fermentation cycles were performed in duplicate (strawberry initial sample and strawberry wine).

Acetic acid fermentation

An active mixture of acetic acid bacteria from a Frings acetator (Heinrich Frings Gmb & Co., KG, Bonn, Germany) (Baena-Ruano and others 2006, 2010 a, b; Garcia-Garcia and others 2007; Jimenez-Hornero and others 2009 a, b; Maestre and others 2008) producing alcohol vinegar was used as started for the acetification process. The fermentation was operated in a semi-continuous mode under the following operational conditions: temperature, 31 °C; agitation, 500 rpm; and dissolved oxygen, 70%. Initially, the bioreactor was loaded with 3.6 L of alcoholic fermented strawberry; once ethanol was depleted, 2.6 L of acetic acid ferment were unloaded and subsequently replaced by the same volume of alcoholic fermented strawberry. Two fermentation cycles were performed in duplicate (strawberry vinegar).

Samples

Samples, which were supplied by Department of Inorganic Chemistry and Chemical Engineering, Faculty of Sciences, University of Cordoba (Cordoba, Spain), were taken at the beginning (strawberry initial samples) and at the end of each fermentation cycle (final samples), in both fermentation processes.

Sample preparation

The preparation of anthocyanin fraction of fermented strawberry was carried out according to Cerezo (2010). An Amberlite XAD7HP column (30 x 1.5 cm) was conditioned with 200 mL of methanol and then 200 mL of water. A total of 20 g of sample was diluted with water (1:1 w/v). The column was loaded with the diluted sample and cleaned with water. Subsequently, the anthocyanin fraction was eluted with methanol: acetic acid (19:1); flow rate 1 drop/s. This fraction was collected and concentrated with a rotary evaporator under vacuum (Büchi Rotavapor, R-200/205, Flawil, Switzerland). Finally the extracts, were reconstituted in 2 mL of acidified water (5% formic acid) and stored at -20 ºC until analysis.

Determination of Total Phenolic Index (TPI)

The TPI was determined by the Folin–Ciocalteu method (Klopotek and others 2005). Firstly, 50 µL of each sample were diluted in 1 mL of distilled water. Then, 20 µL of sample solution was mixed with 1.58 mL of distilled water in a glass cuvette. Subsequently, 100 µL of Folin-Ciocalteu's reagent were added. 4 min after, we put in 300 µL of Na_2CO_3 (20%). After the incubation for 30 min in a bath at 40 ºC, the absorbance was recorded at 750 nm. The TPI was expressed as gallic acid equivalents using this compound as standard. Samples were analyzed in quadruplicate.

158 **Determination of Total Anthocyanin (TA) content**

159 The TA content was estimated by a pH differential method (Giusti and Wrolstad 2001).
160 Firstly, samples were filtered and diluted (1/5). Then, 600 µL of each sample were
161 mixed with 2.4 mL of sodic acetate (pH 4.5) or potassium chloride buffer (pH 1). After
162 15 min, absorbance (A) was measured at 520 and 700 nm in both buffers (pH 1.0 and
163 4.5). Samples were analyzed in quadruplicate.

164 Results were calculated as follows:

165 $A = (A_{515} - A_{700})_{pH1.0} - (A_{515} - A_{700})_{pH4.5}$

166 C (mg/L) = (A x molecular weight x dilution factor x 1000)/ ε l

167 The molecular weight of pelargonidin 3-glucoside is 433.0 g/mol and ε is 22400 mol^{-1}.
168 1= pathlenght (1cm).

169 **Color measurements**

170 Color measurements were determined using a Konica Minolta CM-3600d
171 spectrophotometer (Minolta Co. Ltd., Osaka, Japan) in the CIELab color space, with the
172 D65 illuminant and 10º observer. Color results were expressed as tristimulus
173 parameters (L^*, a^*, b^*, H^*, C^*). Hue angle ($H^* = \tan^{-1} b^*/a^*$) indicates sample color (0° or
174 360° = red, 90° = yellow, 180° = green, 270° = blue), and chroma ($C^* = [a^{*2} + b^{*2}]^{1/2}$)
175 indicates color purity or saturation (high values are more vivid); a^* and b^* chromaticity
176 coordinates indicate color directions green ($-a^*$)/red ($+a^*$) and blue ($-b^*$)/yellow ($+b^*$)
177 (Bakker and others 1986). Samples were analyzed in quadruplicate.

178 **Analysis of individual anthocyanin compounds**

An UHPLC Dionex Ultimate 3000 system (ThermoScientific, San Jose, USA), coupled to a Thermo Scientific Q-ExactiveTM hybrid quadrupole-orbitrap mass spectrometer (Bremen, Germany), was used. The UHPLC system consisted of consisting of a quaternary Rs Pump Dionex Ultimate 3000 (serial number: 8077352) and Rs autosampler Dionex Ultimate 3000 (serial number 8077399), connected to a quadrupole-orbitrap (Q Exactive) hybrid mass spectrometer with heated-electrospray ionization probe (HESI-II, Thermo Fisher Scientific, Bremen, Germany). The analytical method was previously published (Natić and others 2015). Separation was performed on a column SB-C18 (2.1 x 100 mm, 1.8 µm) (Agilent, USA). Injection volume was 1 µL and flow rate was 0.4 mL/min. The solvents used as a mobile phase were: Solvent A (water/formic acid 95:5 v/v) and solvent B (acetonitrile/formic acid 95:5 v/v), scheduled in the following gradient: 0.0-2.0 min 5% B, 2.0-12.0 min from 5% to 100 % B, 12.0-13.0 min from 100% to 5% B, then 5% B up to 15.0 min. Anthocyanin identification and quantitation were acquired in positive mode by full-range acquisition covering *m/z* 100-1500 at 35,000 resolution and by targeted MS^2 normalized Higher Energy Collision Dissociation (HCD). HESI source parameters were as follows: cell at 20 eV, source voltage 3.5 kV, tube lens voltage 50 V, capillary temperature 263 ºC, and sheath and auxiliary gas flow rate (N_2) 50 and 13 (arbitrary units). Xcalibur software (version 3.0.63) was used for instrument control, data acquisition and data analysis. Compounds were identified according to their, calculated mass, accurate mass, mass spectra, characteristic fragmentation data and retention time. Pelargonidin 3-glucoside, cyanidin 3-glucoside, delphinidin 3-glucosise and peonidin 3-glucoside available standards were used both for positive identification

and quantification purposes. Anthocyanin compounds were quantified using the areas of the aglycone counterparts.

Statistical analysis

One-way analysis of variance (ANOVA test) ($p< 0.005$) was used to explore significant differences in anthocyanin composition between initial, wine and vinegar strawberry samples using statistical software (StatSoft Inc 2004). Multivariate statistical analysis was used to interpret data.

Results and discussion

Identification of anthocyanins in fermented products

Table 1 summarizes the retention time (min), molecular formula (M^+), calculated and accurate mass (*m/z*), accuracy error (ppm) and MS/MS fragmentation used to identify a total of 19, 18 and 14 anthocyanin compounds by (UHPLC-MS/MS) Q Exactive in the strawberry initial sample (initial point previous to the fermentation), strawberry wine and strawberry vinegar, respectively. Figure 1 shows UHPLC-MS chromatogram profile of the strawberry initial sample which include: 14 pelargonidin derivates, 3 cyanidin derivates, 1 delphinidin derivate and 1 peonidin derivate.

As expected, pelargonidin 3-glucoside (peak 8), pelargonidin 3-rutinoside (peak 10) and cyanidin 3-glucoside (peak 6) were identified in the strawberry initial sample according to anthocyanin composition of strawberry fruit (Lopes-da-Silva and others 2002; Cerezo and others 2010). These compounds were also identified in the strawberry wine and vinegar samples (Table 1). They have also been described on other products made from strawberry: jams (Amaro and others 2013), spreads

224 (Kadivec and others 2013), strawberry juice model (Díaz-García and others 2009),
225 juices (Oszmiański and Wojdyio 2009; Garzón and Wrolstad 2002), pasteurized
226 strawberry (Oliveira and others 2014) and gluconic fermented strawberry beverage
227 (Hornedo-Ortega 2016 a, b).

228 Four anthocyanin compounds were tentatively identified with high accuracy for the
229 first time to be present in the beverages obtained by alcoholic fermentation. This is the
230 case of: pelargonidin 3-sambubioside (peak 9), pelargonidin dissacharide (hexose +
231 pentose) acylated with acetic acid (peaks 12, 14 and 15), cyanidin 3-(6-acetyl)-
232 glucoside (peak 17) and pelargonidin 3-(6-succinyl)-arabinoside/3-(6-malonyl)-
233 rhamnoside (peak 19).

234 Eight anthocyanin compounds were tentatively identified with high accuracy for the
235 first time to be present in the beverages obtained by acetic fermentation of strawberry
236 as follows: catechin-(4-8)-pelargonidin 3-glucoside (peak 1), afzelechin-pelargonidin 3-
237 glucoside and Epi-afzelechin-pelargodinin 3-glucoside (peak 2 and 4 respectively),
238 delphinidin 3-glucoside (peak 5), pelargonidin 3-sambubioside (peak 9), 5-
239 carboxypyranopelargonidin 3-glucoside (peak 13), pelargonidin 3- (6-acetyl)-glucoside
240 (peak 18) and pelargonidin 3- (6-succinyl)-arabinoside/3-(6-malonyl)-rhamnoside (peak
241 19).

242 Anthocyanins compounds identified in both strawberry wine and vinegar were:
243 catechin-(4-8)-pelargonidin 3-glucoside, afzelechin-pelargonidin 3-glucoside, epi-
244 afzelechin-pelargonidin 3-glucoside and pelargonidin dissacharide (hexose + pentose)
245 acylated with acetic acid. Three pelargonidin dissacharide (hexose + pentose) acylated
246 derivatives were detected (at different retention times, exact mass *m/z* 607.1657 and

247 main fragments *m/z* 271.0600). Likely, this could be explained by the presence of
248 different sugar substituents or different linkages between the pentose and hexose
249 residues in each of the anomeric carbons of the pigments.

250 5-carboxypyranopelargonidin 3-glucoside was present in the strawberry initial samples
251 and in the strawberry wine and vinegar (Table 1). This pigment has been reported
252 previously in strawberry fruit (Andersen and others 2004; Lopes-da-Silva and others
253 2007; Cerezo and others 2010) and recently in gluconic fermented beverage made
254 from strawberry (Hornedo-Ortega and others 2016 b). However, this is the first time
255 that 5-carboxypyranopelargonidin 3-glucoside is reported in strawberry vinegar (Figure
256 2). There is evidence of the appearance of pyranoanthocyanin pigments in other red
257 fruit derived product such as cherry juices (5-carboxypyrano-cyanidin 3-(2-
258 glucosylrutinoside)) as a result of the storage period (Bonerz and others 2007) and also
259 in red wine due to the fermentation and ageing processes (González-Paramás and
260 others 2006).

261 Pelargonidin 3-malonylglucoside was previously found in strawberry fruit (Cerezo and
262 others 2010; Buendía and others 2010) and in pomace, spreads and juices made from
263 strawberry (Šaponjac and others 2015; Kadivec and others 2013; Oszmiański and
264 others 2009). Pelargonidin 3-sambubioside has been recently reported in gluconic
265 fermented strawberry beverage (Hornedo-Ortega and others 2016b). However, this is
266 the first time it is found in strawberry wine and vinegar (Figure 3).

267 Cyanidin 3-rutinose has been identified in sour cherry (*Prunus cerasus L*) juice (Díaz-
268 García and others 2013). Moreover, Sokól-Letowska (2014) identified this compound
269 with high accuracy (*m/z* 595.1668) in blackcurrant, chokeberry, Mahonia, raspberry,

Sole, Sour cherry and strawberry liqueurs. Furthermore, cyanidin 3-(6”-acetyl)-glucoside has been previously described in six Chilean berry extracts (Brito and others 2014). Additionally, this compound has been identified in the skin and wine made from *Vitis vinifera L.* (cv. *Aglianico*) grapes (De Nisco and others 2013).

Finally, delphinidin 3-glucoside has been also reported in strawberry fruit (Cerezo and others 2010) in strawberry bilberry juice (Díaz-García and others 2013) and in gluconic and alcoholic fermented strawberry products (Hornedo-Ortega and others 2016 a, b). Table 1 shows its evidence in vinegars made from strawberry as a novel contribution of this paper.

Changes in anthocyanin concentrations due to the fermentation processes

Table 2, shows the concentrations (µg/L) of the strawberry initial sample, the strawberry wine and the strawberry vinegar samples. All anthocyanin compounds decreased after both fermentation processes, being the higher losses due to the acetic fermentation (91%) rather than to the alcoholic one.

The anthocyanins compounds with the greatest concentration in our samples were: pelargonidin 3-glucoside, pelargonidin 3-rutinoside, cyanidin 3-glucoside according other authors (Lopes-da-Silva and others 2007; Cerezo and others 2010) followed by pelargonidin 3-malonylglucoside and pelargonidin 3-(6-acetyl)-glucoside.

Specifically, after alcoholic fermentation process the major compound that underwent the highest loss in its concentration was cyanidin 3-glucoside that decreased about 40% followed by pelargonidin 3-glucoside (20.4%). Pelargonidin and cyanidin have a great number of hydroxyl groups that decreases the stability comparing with other

anthocyanin with methyl groups (Brouillard 1983). Furthermore the monoglycoside anthocyanins are more unstable that di-tri-glycosides ones.

In contrast, pelargonidin 3-rutinoside maintained its concentration. Oliveira (2014) also reported that pelargonidin 3-rutinoside is preserved after a strawberry pasteurization treatment.

On the other hand, the acylated anthocyanins suffer much minor losses than other compounds. It is well known that anthocyanins with acylating substituents are more stable during processing than other natural pigments (Cevallos-Casals and others 2004; Giusti and Wrolstad 2003). This stabilization has been attributed to the stacking of the acyl group with the pyrylium ring of the flavynium cation, by reducing the susceptibility of nucleophile attack of water and the corresponding formation of a pseudobase or a chalcone (Brouillard and others 2003).

Other possible fact that explain the decrease of the anthocyanin compounds can be the adsorption mechanism between the yeast (*S. cerevisiae*) and anthocyanins that has been stated as responsible of the decrease on these compounds in alcoholic fermented final products (Morata and others 2003). Acetification process decreased anthocyanin concentration in a larger extent. Cyanidin 3-glucoside was the most affected (97.4%), followed by pelargonidin 3-malonylglucoside (95.3%), pelargonidin 3-glucoside (89.3%), pelargonidin 3-(6-acetyl)-glucoside (89.3%) and pelargonidin 3-rutinoside (85%). Besides, all minor anthocyanin compounds were strongly affected by acetification process (60-96%). Indeed, three anthocyanin compounds disappeared (pelargonidin 3,5-diglucoside, pelargonidin dissacharide (hexose + pentose) acylated with acetic acid and cyanidin 3-(6-acetyl)-glucoside).

315 It must be highlighted that 5-carboxypyranopelargonidin 3-glucoside is the compound
316 that suffers minor losses in this concentration and the most stable in both
317 fermentation processes (16.2 and 60% respectively) in agreement with its reported
318 stability during the storage of gluconic fermented strawberry beverage (Hornedo-
319 Ortega and others 2016b). The pyranoanthocyanins are cycloaddition products, which
320 have an additional pyran ring between the C4 position in the C ring and the hydroxyl
321 group on the C5 position in the A ring of the anthocyanin molecule. These compounds
322 are highly stable and resistant to and oxidative degradation, therefore they can
323 significantly contribute to the color stability (de Freitas and Mateus 2011; Rentzsch and
324 others 2007).

325 **Changes in TPI, TA and color measurements**

326 Table 3 displays pH, TPI, TA and color measurements of strawberry initial sample,
327 strawberry wine and strawberry vinegar.

328 There were not significant changes ($p<0.05$) in pH value between initial sample and
329 strawberry wine. However, a decrease was observed for strawberry vinegar as
330 expected because of the production of acetic acid during acetic fermentation. Despite
331 that the anthocyanin are more stable at acidic pH (predominantly ion flavilium form)
332 (Clifford and others 2000) the decrease in the pH value not prevent to the several
333 effect of the acetic fermentation. In accordance with anthocyanin data discussed in the
334 previous section, TA showed a diminution in the concentration (29 and 80%, wine and
335 vinegar, respectively). Previous studies have reported an important decreased
336 between 63-85% in TA after strawberry wine production and between 97.2-99% after
337 acetification process (Ubeda and others 2013; Klopotek and others 2005). Similarly,

338 derivatives products made from other red fruits such as *S. cerevisiae* fermented
339 pomegranate product showed a dramatic decreased in TA (90.2%) due to alcoholic
340 fermentation process (Ordoudi and others 2014). Similarly, other process such as the
341 production of strawberry jams also produces accused losses in the anthocyanin
342 composition. In this way, a few quantities or the absence of anthocyanins has been
343 described in commercial strawberry jams (Da Silva-Pinto and others 2007).

344 The color of the initial strawberry sample and the strawberry wine and vinegar was
345 evaluated using the CIELab parameters (*L**, *a**, *b**, *C** and *H** values), which presented
346 statistical differences with both fermentation processes (Table 3). The *L** value,
347 corresponding to luminosity of samples increased, being brighter for strawberry
348 vinegar than for strawberry wine. Additionally, we observed an increase in *a** value
349 corresponding to a higher intensity of the red color. However, after acetic acid
350 fermentation this parameter decreased significantly. *b** value increased with
351 fermentation process that indicated the presence of orange-yellow constituents. For
352 Chroma value (*C**) we found a very high saturation in the color after both processes
353 being higher for alcoholic samples. *H** value increased with the subsequent
354 fermentation processes indicating a change of red color (*H**=0) to yellow color
355 (*H**=90). Strawberry vinegar showed a range color between red-orange.

356 Multivariate statistical analysis showed that color is correlated with anthocyanin
357 composition. Indeed, correlations higher that 0.91 were obtained for 12 out of 17
358 anthocyanins on *L** and *a** parameters (Table 4).

359 In fact, 100% of the samples could be classified into their groups (strawberry initial
360 sample, strawberry wine and strawberry vinegar) by Linear Discriminant Analysis

(LDA). Two analyses were performed achieving identical results. The first one included color parameters, the second the major anthocyanins compounds (pelargonidin 3-glucoside, pelargonidin 3-rutinoside and cyanidin 3-glucoside).

These results confirm that alcoholic and acetic fermentations have a crucial influence on the anthocyanin composition and color properties of the beverages.

Conclusion

In conclusion, this study reported with high accuracy the identification for the first time of 4 and 8 anthocyanin compounds in wine and vinegar made from strawberry respectively. Alcoholic fermentation affected moderately anthocyanin compounds (19% of decrease). However, acetic fermentation process produced higher losses on the anthocyanin composition. Moreover, 5-carboxypyranopelargonidin 3-glucoside was the anthocyanin compound affected in minor degree by both fermentation processes. The color of the wine and the vinegar made from strawberry changed during the fermentation process, varying from red to orange color.

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383 **Author Contributions**

384 R.Hornedo-Ortega performed the experiments, collected and analyzed all data,
385 interpreted the results and first drafted of the manuscript. M.A. Álvarez-Fernández
386 participated in UHLPC- MS/MS analysis. A.B. Cerezo revised the results. I. Garcia-Garcia
387 supplied strawberry fermented samples. A.M. Troncoso and M.C. Garcia-Parrilla
388 created the idea of this work and revised the manuscript.

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Table 1: Anthocyanins found in strawberry initial sample and strawberry wine and vinegar samples in positive ionization mode. Mean expected retention time (Rt) (min), molecular formula, calculated mass (*m/z*), accurate mass (*m/z*), accuracy error (ppm), and MS/MS fragmentation.

Peaks	Rt	Compounds	Molecular formula (M^+)	Calculated mass (*m/z*)	Accurate mass (*m/z*)	Error (ppm)	MS/MS Fragmentation	Strawberry Initial Sample	Strawberry wine	Strawberry vinegar
1	3.80	Catechin-(4-8)-pelargonidin 3-glucoside	$C_{36}H_{33}O_{16}^+$	721.1763	721.1755	-1.1652	559.1232 / 407.07561 / 313.0708	X	X	X[a]
2	4.54	Afzelechin-pelargonidin 3-glucoside	$C_{36}H_{33}O_{15}^+$	705.1814	705.1803	-1.5778	543.1281 / 407.0755 / 313.0703	X	X	X[a]
3	4.58	Pelargonidin 3,5-diglucoside	$C_{27}H_{31}O_{15}^+$	595.1657	595.1657	-0.0494	433.1146 / 271.0600	X	X	-
4	4.69	Epi-Afzelechin-pelargonidin 3-glucoside	$C_{36}H_{33}O_{15}^+$	705.1814	705.1806	-1.0749	543.1282/ 407.0755 / 313.0705	X	X	X[a]
5	4.73	Delphinidin 3-glucoside	$C_{21}H_{21}O_{12}^+$	465.1028	465.1003	-1.8261	303.0140	X	X	X[a]
6	4.73	Cyanidin 3-glucoside	$C_{21}H_{21}O_{11}^+$	449.1078	449.1063	-3.5280	287.05502	X	X	X
7	4.80	Cyanidin 3-rutinoside	$C_{27}H_{31}O_{15}^+$	595.1657	595.1651	-1.1339	449.1071 / 287.0551	X	X	X
8	4.95	Pelargonidin 3-glucoside	$C_{21}H_{21}O_{10}^+$	433.1129	433.1121	-1.9546	271.0599	X	X	X
9	4.97	Pelargonidin 3-sambubioside	$C_{26}H_{29}O_{14}^+$	565.1552	565.1538	-2.6319	271.0599	X	X[a]	X[a]
10	5.03	Pelargonidin 3-rutinoside	$C_{27}H_{31}O_{14}^+$	579.1708	579.1700	-1.3635	433.1129/271.0601	X	X	X
11	5.10	Peonidin 3-glucoside	$C_{22}H_{23}O_{11}^+$	463.1235	463.1208	-5.6967	301.0706	X	-	-
12	5.14	Pelargonidin dissacharide (hexose + pentose) acylated with acetic acid	$C_{28}H_{31}O_{15}^+$	607.1657	607.1663	0.9454	271.0600	X	X[a]	-
13	5.14	5-carboxypyranopelargonidin 3-glucoside	$C_{24}H_{21}O_{12}^+$	501.1028	501.1035	1.4621	339.0497	X	X	X[a]
14	5.29	Pelargonidin dissacharide (hexose + pentose) acylated with acetic acid	$C_{28}H_{31}O_{15}^+$	607.1657	607.1659	0.2421	271.0600	X	X	-
15	5.40	Pelargonidin dissacharide (hexose + pentose) acylated with acetic acid	$C_{28}H_{31}O_{15}^+$	607.1657	607.1655	-0.3610	271.0600	X	X	X
16	5.40	Pelargonidin 3-malonylglucoside	$C_{24}H_{23}O_{13}^+$	519.1133	519.1125	-1.4837	271.0599	X	X	X
17	5.49	Cyanidin 3-(6-acetyl)-glucoside	$C_{23}H_{23}O_{12}^+$	491.1184	491.1161	-4.1663	287.0551	X	X[a]	-
18	5.73	Pelargonidin 3-(6-acetyl)-glucoside	$C_{23}H_{23}O_{11}^+$	475.1235	475.1224	-2.3413	271.0596	X	X	X[a]
19	6.29	Pelargonidin 3-(6-succinyl)-arabinoside/ 3-(6-malonyl)-rhamnoside	$C_{25}H_{27}O_{11}^+$	503.1548	503.1537	-2.1972	271.0599	X	X[a]	X[a]

[a] anthocyanin compounds reported for the first time in wine and vinegar made from strawberry

Table 2: Anthocyanin concentrations (µg/L) in strawberry initial sample strawberry wine and strawberry vinegar

Compounds	Strawberry initial sample	Strawberry wine	Strawberry vinegar
Catechin-(4-8)-pelargonidin 3-glucoside	6.601 ± 0.194	3.748 ± 0.116[a]	0.501 ± 0.006[b]
Afzelechin-pelargonidin 3-glucoside	7.647 ± 0.000	4.503 ± 0.003[a]	0.249 ± 0.002[b]
Pelargonidin 3,5-diglucoside	0.598 ± 0.013	0.786 ± 0.018[a]	-
Epi-Afzelechin-pelargonidin 3-glucoside	3.540 ± 0.002	0.865 ± 0.150[a]	0.232 ± 0.002[b]
Delphinidin 3-glucoside	NC	NC	NC
Cyanidin 3-glucoside	71.180 ± 2.667	42.714 ± 0.427[a]	1.096 ± 0.027[b]
Cyanidin 3-rutinoside	2.372 ± 0.011	1.925 ± 0.021	0.348 ± 0.002[b]
Pelargonidin 3-glucoside	1060.078±7.746	844.194 ± 2.780[a]	90.223 ± 0.611[b]
Pelargonidin 3-sambubioside	0.771 ± 0.002	1.266 ± 0.053[a]	NC
Pelargonidin 3-rutinoside	138.815 ± 1.247	140.725 ± 1.087	21.203 ± 0.095[b]
Peonidin 3-glucoside	NC	-	-
Pelargonidin dissacharide (hexose + pentose) acylated with acetic acid	0.454 ± 0.006	0.843 ± 0.145[a]	-
5-carboxypyranopelargonidin 3-glucoside	20.938 ± 0.474	17.545 ± 0.144[a]	7.008 ± 0.071[b]
Pelargonidin dissacharide (hexose + pentose) acylated with acetic acid	1.038 ± 0.001	0.921 ± 0.003[a]	-
Pelargonidin dissacharide (hexose + pentose) acylated with acetic acid	0.593 ± 0.001	0.588 ± 0.005	0.053 ± 0.000[b]
Pelargonidin 3-malonylglucoside	57.631 ± 0.508	47.416 ± 0.095[a]	2.245 ± 0.011[b]
Cyanidin 3-(6-acetyl)-glucoside	1.429 ± 0.029	1.302 ± 0.024[a]	-
Pelargonidin 3-(6-acetyl)-glucoside	51.452 ± 1.041	45.321 ± 0.183[a]	4.848 ± 0.016[b]
Pelargonidin 3-(6-succinyl)-arabinoside/3-(6-malonyl)-rhamnoside	13.661 ± 0.224	11.486 ± 0.119[a]	0.459 ± 0.003[b]
Total	**1438.798**	**1166.148**	**128.466**

[a] significant differences between strawberry initial sample and strawberry wine $p<0.05$.

[b] significant differences between strawberry wine and strawberry vinegar $p<0.05$.

Table 3: pH, TPI (mg/L), TA (mg/L) and color measurements. All measures were analyzed in quadruplicate.

Samples	pH	TPI	TA	*L**	*a**	*b**	*C**	*H**
Strawberry initial sample	3.32	4.47 ± 1.57	94.86 ± 5.13	42.87 ± 0.04	63.68 ± 0.10	61.36 ± 0.23	88.43 ± 0.22	43.94 ± 0.06
Strawberry wine	3.29	2.29 ± 0.08	67.61 ± 6.36[a]	46.49 ± 0.03[a]	65.88 ± 0.02[a]	79.45 ± 0.07[a]	103.21 ± 0.05[a]	50.33 ± 0.02[a]
Strawberry vinegar	2.72	2.47 ± 0.31	18.61 ± 3.35[b]	62.61± 0.02[b]	55.14 ± 0.01 [b]	73.61 ± 0.07[b]	91.97 ± 0.05[b]	53.16 ± 0.03[b]

[a] significant differences between initial sample and strawberry wine $p<0.05$.

[b] significant differences between strawberry wine and strawberry vinegar $p<0.05$.

L*: lightness.

a* and b* chromaticity coordinates indicate color directions: green (−a*)/red (+a*) and blue (−b*)/yellow (+b*).

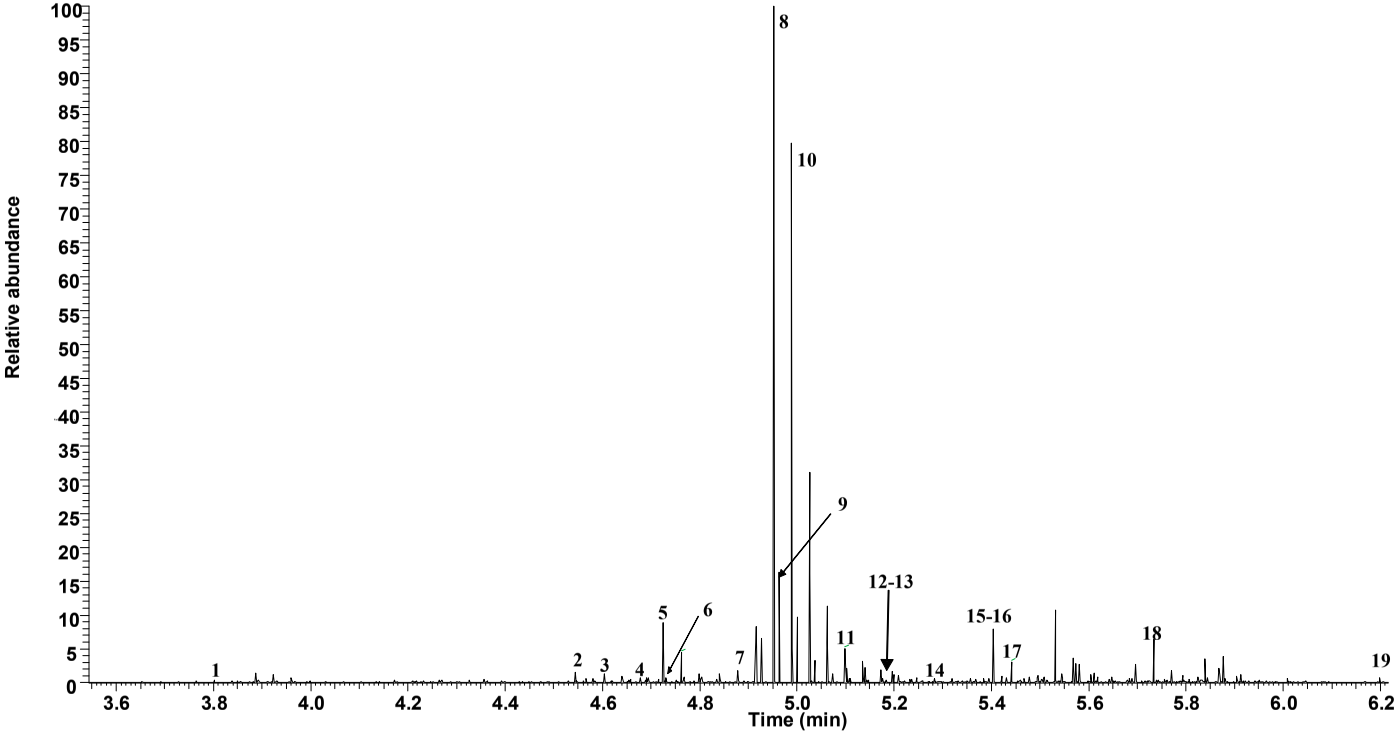
C* = [a*2 + b*2]1/2 indicates color saturation.

H* = tan−1 b*/a* indicates sample color.

Table 4: Linear correlations between anthocyanin composition and color parameters.

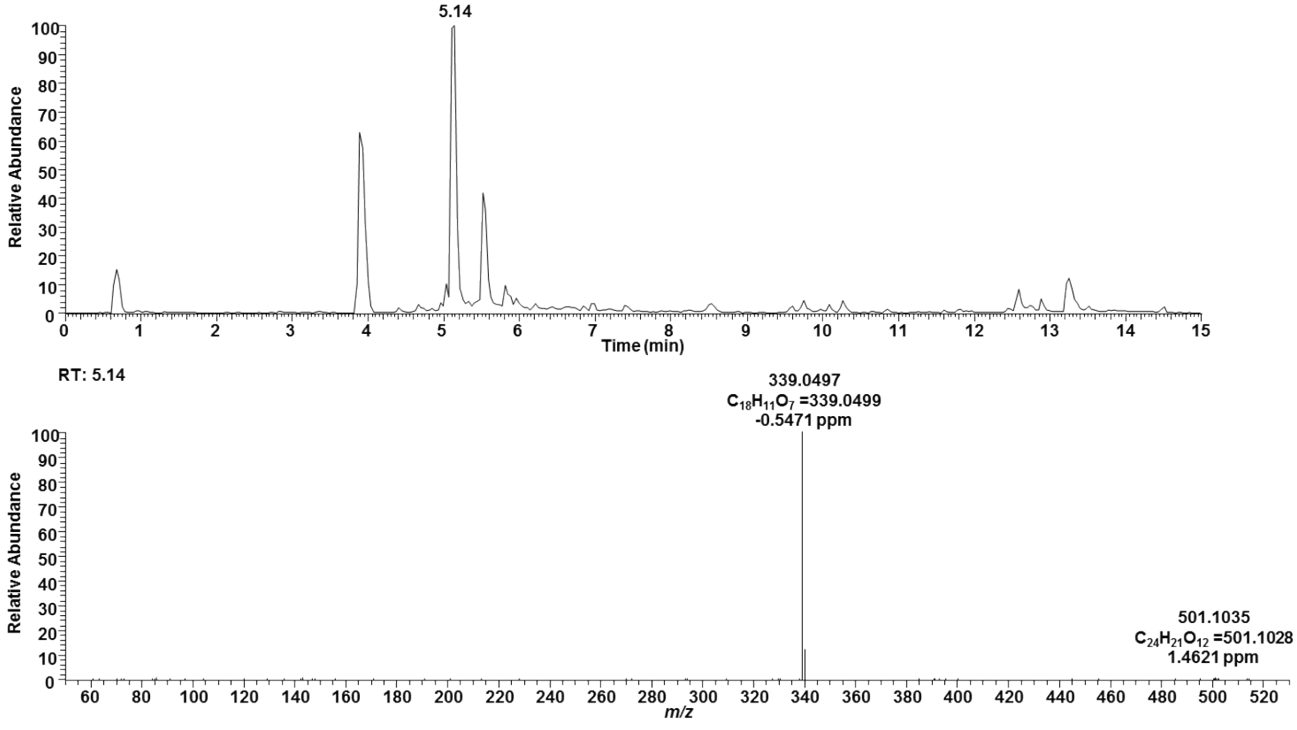
Compounds	***L****	***a****	***b****	***C****	***H****
Catechin-(4-8)-pelargonidin 3-glucoside	-0.95	0.77	-0.63	-0.19	-0.97
Afzelechin-pelargonidin 3-glucoside	-0.97	0.80	-0.59	-0.15	-0.95
Pelargonidin 3.5-diglucoside	-0.92	1.00	0.03	0.50	-0.56
Epi-Afzelechin-pelargonidin 3-glucoside	-0.77	0.48	-0.87	-0.54	-0.99
Cyanidin 3-glucoside	-0.97	0.82	-0.58	-0.12	-0.94
Cyanidin 3-rutinoside	-0.43	0.73	0.68	0.94	0.12
Pelargonidin 3-glucoside	-0.84	0.98	0.20	0.64	-0.41
Pelargonidin 3-sambubioside	-1.00	0.92	-0.40	0.08	-0.86
Pelargonidin 3-rutinoside	-0.98	0.98	-0.18	0.30	-0.72
Pelargonidin dissacharide (hexose + pentose) acylated with acetic acid	-0.78	0.95	0.27	0.69	-0.33
5-carboxypyranopelargonidin 3-glucoside	-1.00	0.91	-0.42	0.06	-0.87
Pelargonidin dissacharide (hexose + pentose) acylated with acetic acid	-1.00	0.95	-0.30	0.19	-0.80
Pelargonidin dissacharide (hexose + pentose) acylated with acetic acid	-0.99	0.98	-0.21	0.28	-0.74
Pelargonidin 3-malonylglucoside	-1.00	0.93	-0.36	0.12	-0.84
Cyanidin 3-(6-acetyl)-glucoside	-1.00	0.96	-0.27	0.21	-0.78
Pelargonidin 3-(6-acetyl)-glucoside	-1.00	0.95	-0.31	0.17	-0.81
Pelargonidin 3-(6-succinyl)-arabinoside/3-(6-malonyl)-rhamnoside	-1.00	0.94	-0.34	0.14	-0.83

Figure 1.



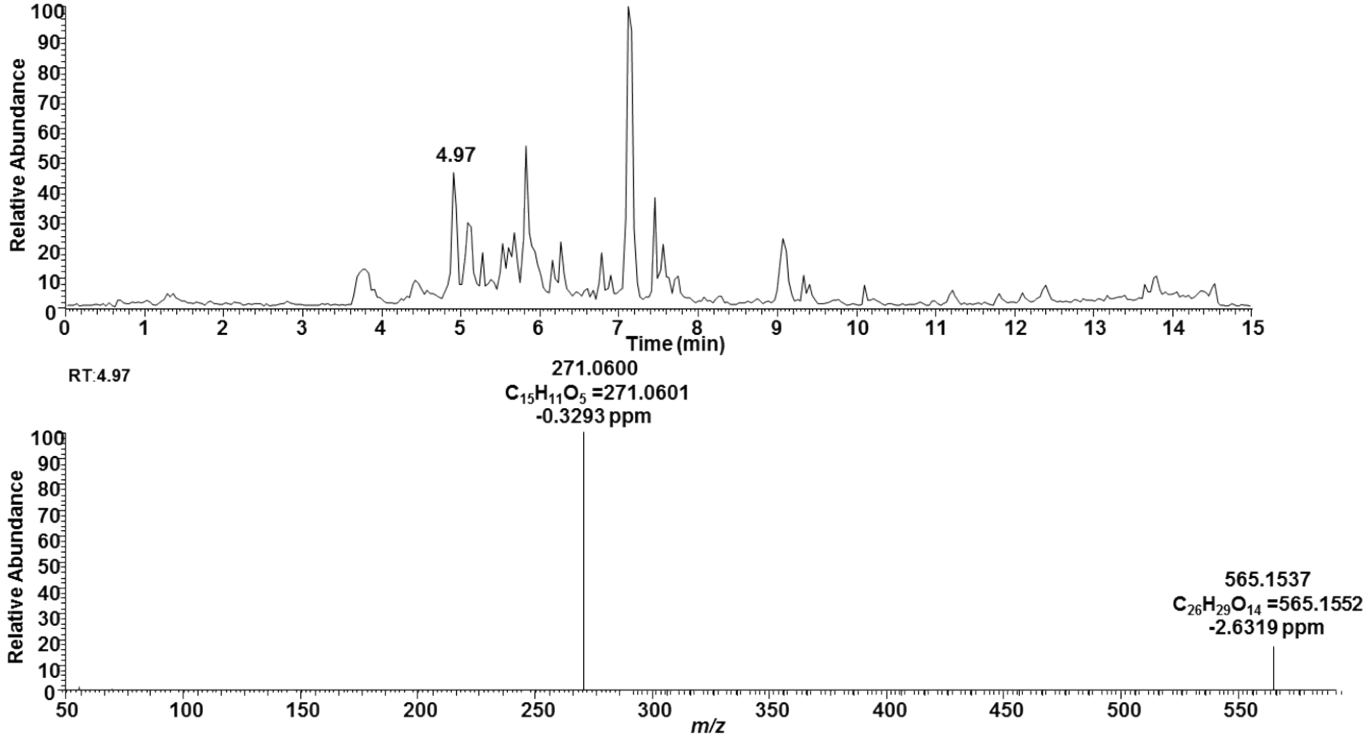
Ion chromatogram from targeted MS2 experiments. The number indicates the compounds shown in Table 1.

Figure 2.



Extracted Ion Chromatogram (XIC) from targeted-MS2 experiment selecting m/z 501.10 corresponding to 5- carboxypyranopelargonidin 3-glucoside (upper) and MS2 spectra (down). Reported for the first time in strawberry vinegar.

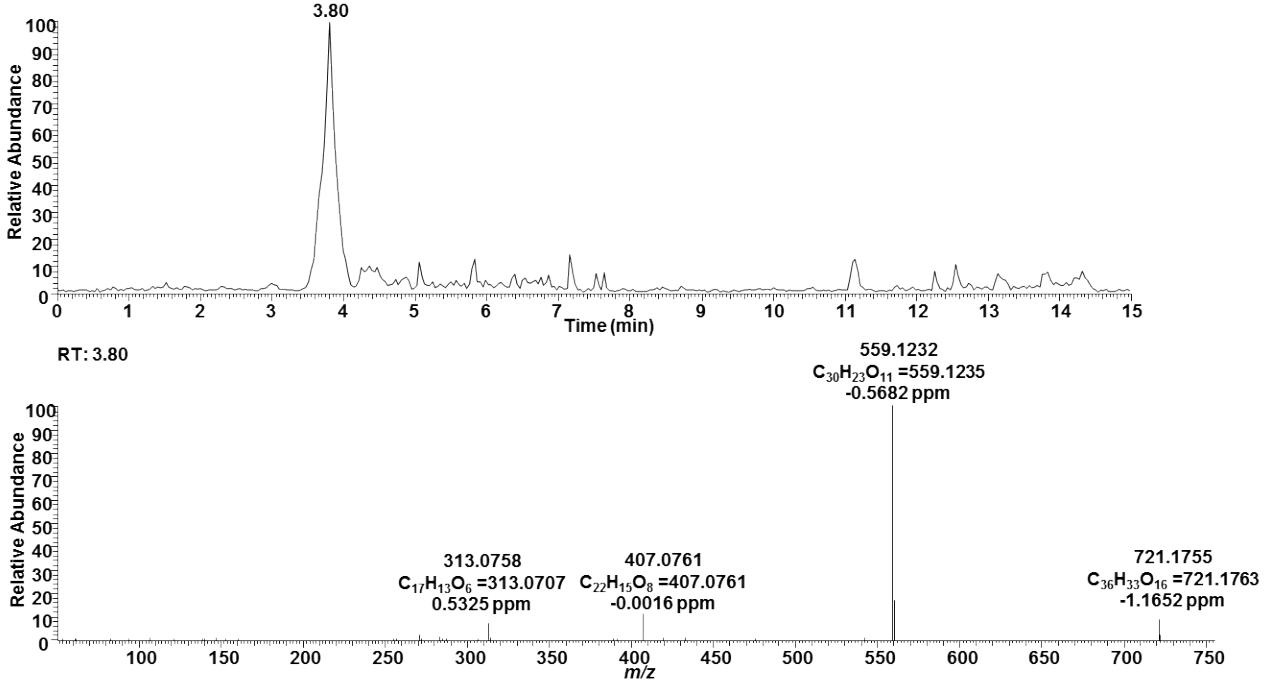
Figure 3.



Extracted Ion Chromatogram (XIC) from targeted-MS2 experiment selecting m/z 565.15 corresponding to pelargonidin 3-sambubioside (upper) and MS2 spectra (down). Reported for the first time in strawberry wine and strawberry vinegar

Supplementary data

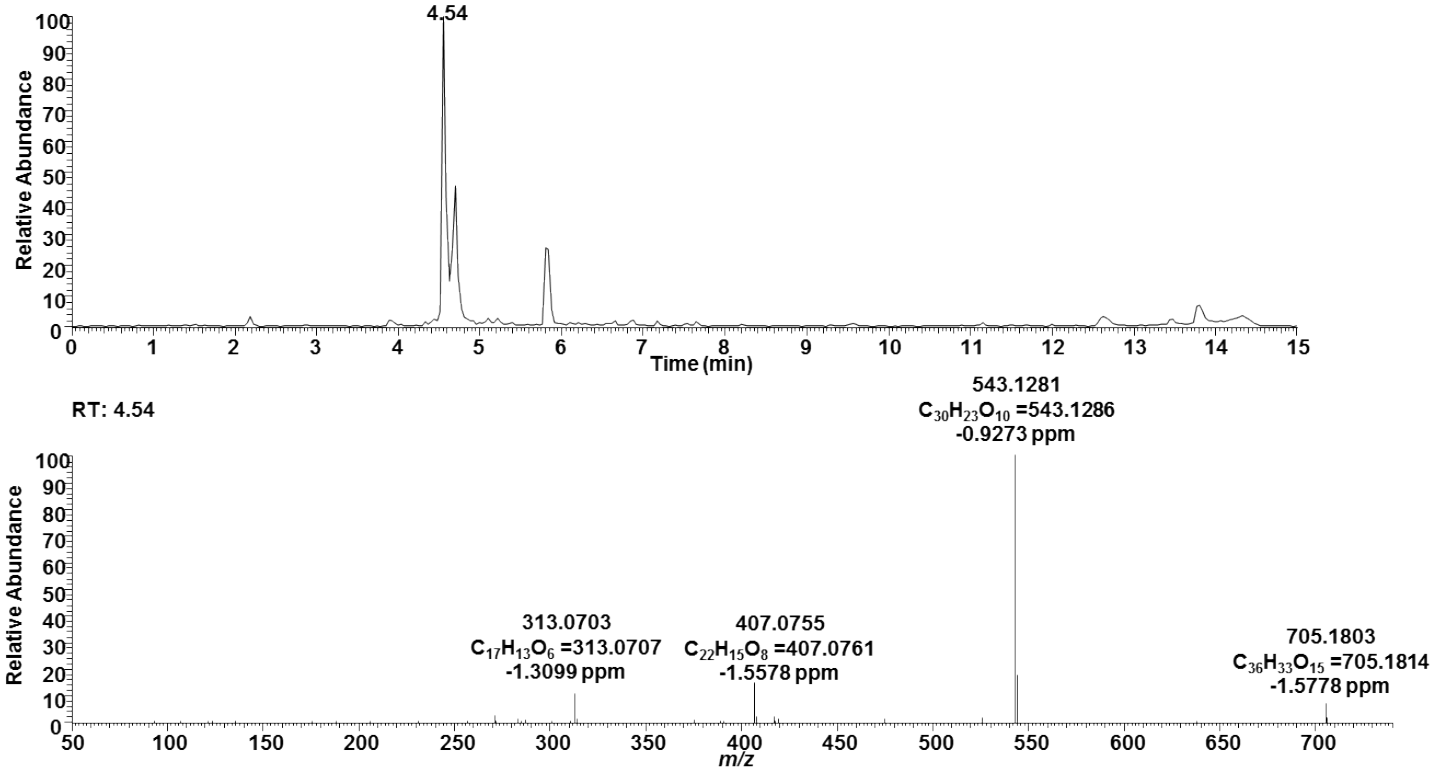
Figura 4.



Extracted Ion Chromatogram (XIC) from targeted-MS2 experiment selecting *m/z* 721.18 corresponding to catechin- (4-8)-pelargonidin 3-glucoside (upper) and MS2 spectra (down). Reported for the first time in strawberry vinegar.

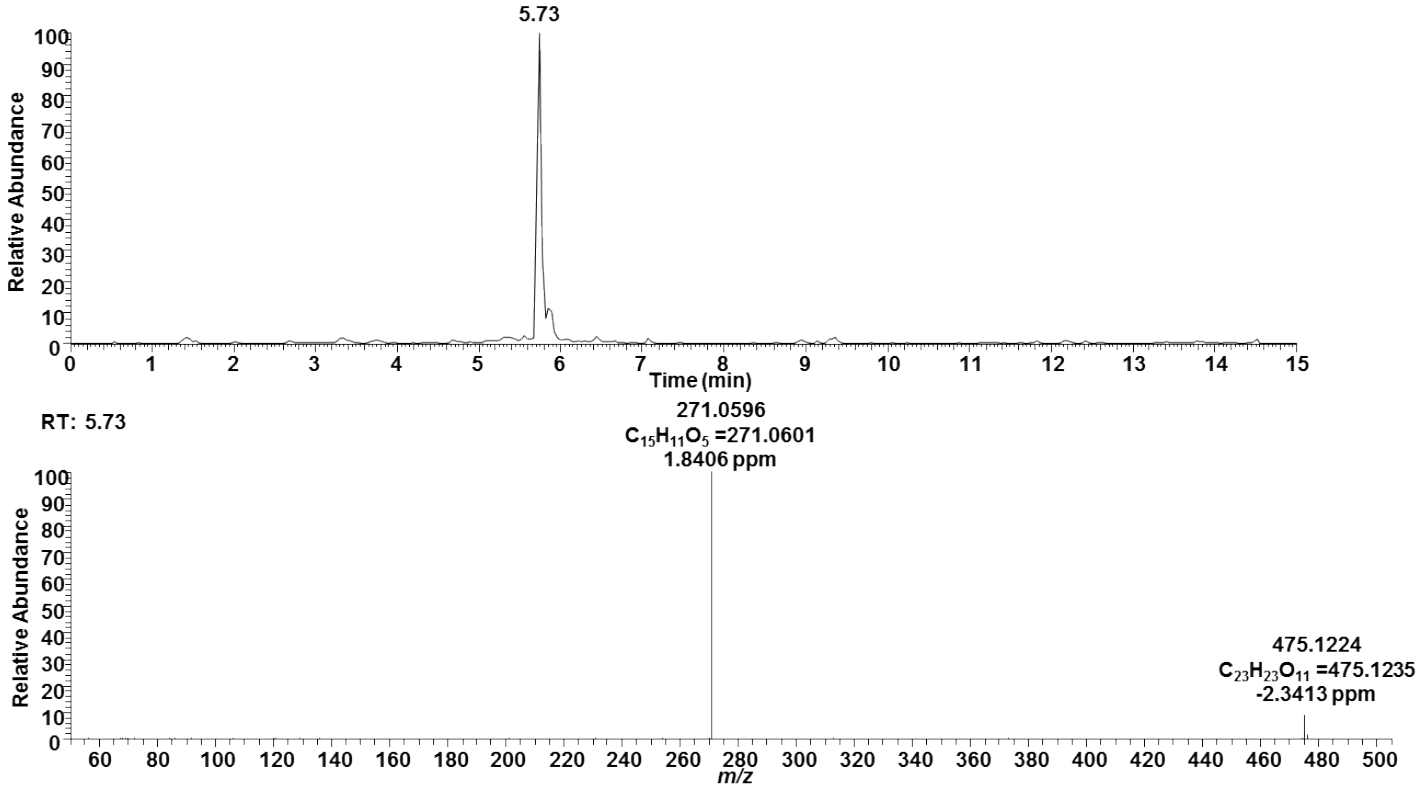
120

Figure 5.



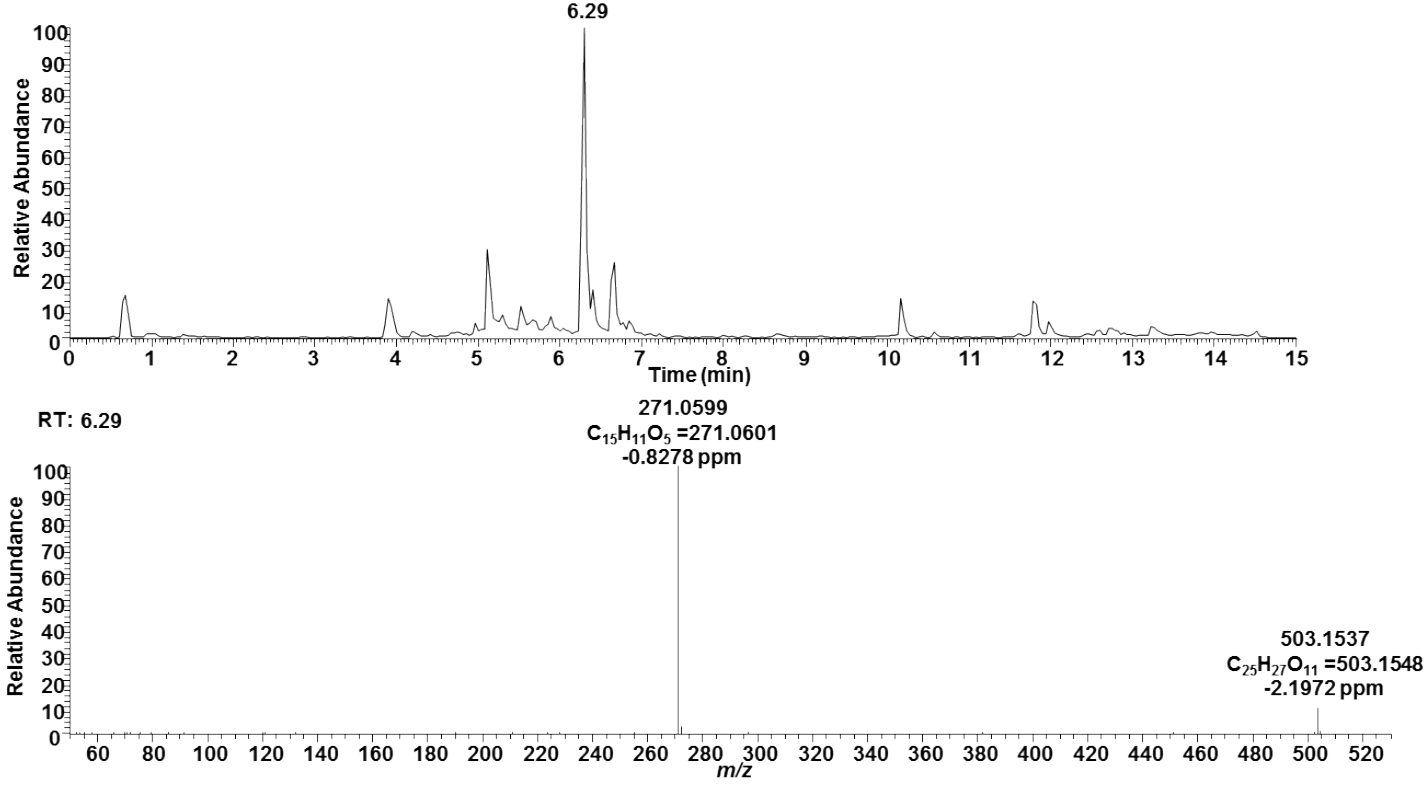
Extracted Ion Chromatogram (XIC) from targeted-MS2 experiment selecting m/z 705.18 corresponding to Afzelechin-pelargonidin 3-glucoside (upper) and MS2 spectra (down). Reported for the first time in strawberry vinegar.

Figure 6.



Extracted Ion Chromatogram (XIC) from targeted-MS2 experiment selecting *m/z* 475.12 corresponding to pelargonidin 3-(6-acetyl)-glucoside (upper) and MS2 spectra (down). Reported for the first time in strawberry vinegar.

Figure 7.



Extracted Ion Chromatogram (XIC) from targeted-MS2 experiment selecting *m/z* 503.15 corresponding to pelargonidin 3-(6-succynil)-arabinoside / 3-(6-malonyl)-rhamnoside (upper) and MS2 spectra (down). Reported for the first time in strawberry wine and strawberry vinegar.

Capítulo 2

Efectos de la fermentación glucónica y alcohólica en la composición antociánica, actividad antioxidante y propiedades neuroprotectoras de bebidas elaboradas a partir de fresa

Effects of gluconic and alcoholic fermentation on anthocyanin composition and antioxidant activity of beverages made from strawberry

Ruth Hornedo-Ortega, Stéphanie Krisa, M.Carmen García-Parrilla and Tristan Richard

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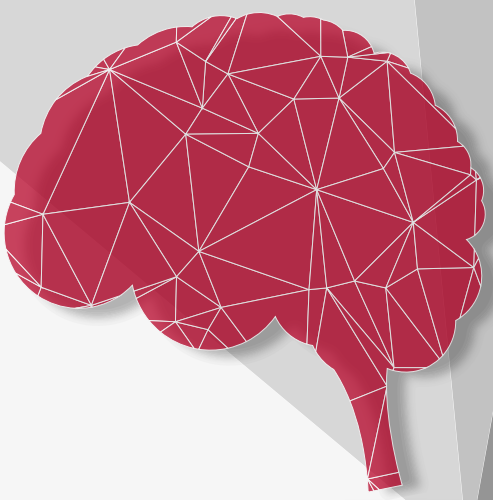

Effects of gluconic and alcoholic fermentation on anthocyanin composition and antioxidant activity of beverages made from strawberry

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Abstract

Strawberry is a very perishable fruit, well-known for being a source of bioactive compounds. The elaboration of the beverages by alcoholic and gluconic fermentation process has been explored as a worthy strategy for preventing food losses as well as preserving bioactive compounds with antioxidant properties.

To this end, this paper aims to characterize the anthocyanin composition of the resulting beverages and to evaluate their antioxidant properties with *in vitro* assays (ORAC, DPPH). Additionally, the protective effect against amyloid-β (Aβ) peptide toxicity in terms of Reactive Oxygen Species (ROS) production and PC12 cells viability was determined.

Eleven anthocyanin compounds were identified and quantified by UHPLC-DAD-MS. Pelargonidin 3-glucoside and its derivatives were the major compounds. Gluconic fermentation preserved anthocyanin composition being an advantage of this innovative process. Accordingly the values of antioxidant activity were higher for gluconic than alcoholic fermented beverages. Indeed, both of them increased cell viability (16 to 57% $p<0.05$) and attenuate the oxidative stress triggered by Aβ (13 to 38 % $p<0.05$).

Keywords: process, drink, polyphenols, ROS, amyloid-β

Pelargonidin 3-glucoside (PubChem CID: 443448); Pelargonidin 3-rutinoside (PubChem CID: 44256626); Pelargonidin 3- (6”-malonylglucoside) (PubChem CID: 45256635); Cyanidin 3-glucoside (PubChem CID: 92131208); Delphinidin 3-glucoside (PubChem CID: 443650).

Abbreviations:

Aβ: amyloid-β

AAPH: 2,2´-diazo-bis-amidinepropane-dihydrochloride

DCFDA: 2',7'-dichlorofluorescin diacetate

DPPH: 2,2-diphenyl-1-picrylhydrazyl

IC_{50}: Half Maximal Inhibitory Concentration

MTT: Thiazolyl Blue Tetrazolium Bromide

ORAC: Oxygen Radical Absorbance Aapacity

ROS: Reactive Oxygen Species

UHPLC: Ultra High-Performance Liquid Chromatography

1. Introduction

Strawberry is an important fruit crop worldwide, especially for fresh consumption. An alternative for avoiding economic loss due to its perishable nature is the elaboration of derivatives products such as jams, yoghourts, products for biscuits or cakes and beverages made from strawberry pureés.

Recently, different studies summarized the evidence for the health benefits of strawberry and other berry fruits (Basu, Nguyen, Betts, & Lyons, 2014; Giampieri et al., 2015). Indeed, strawberry is a good source of bioactive compounds. Furthermore, the antioxidant properties of strawberry have been attributed to its polyphenol and vitamin content, being ascorbic acid, ellagitanins and anthocyanins the greatest contributors to its antioxidant capacity (Aaby, Ekeberg, & Skrede, 2007; Manganaris, Goulas, Vicente, & Terry, 2014). Recently, strawberries were included among the 100 richest sources of dietary polyphenols and also listed in rankings of 89 foods and beverages that provide more than 1 mg of polyphenols per serving (Pérez-Jiménez, Neveu, Vos, & Scalbert, 2010). Anthocyanins are responsible for the red color of berry fruits, such as blueberries, blackberries and strawberries. It is well known that pelargonidin 3-glucoside is the major anthocyanin in strawberry (150–650 mg kg^{-1} of fresh weight) (García-Viguera, Zafrilla, & Tomás-Barberán, 1998; Lopes-da-Silva, Escribano-Bailón, Pérez Alonso, Rivas-Gonzalo, & Santos-Buelga, 2007). Indeed, there are crucial factors that influence significantly the stability of anthocyanin compounds such as process, time, and storage temperature (Clifford, 2000). Several efforts have been done to diminish the effect of process in the composition of products made from

strawberry using different production systems and the employment of modified atmosphere in the storage (Fan et al., 2012; Oliveira et al., 2015).

Furthermore, the production of strawberry drinks is an innovative trend. In particular, the fermentation by *Gluconobacter japonicus* which transforms the glucose content of the fruit into gluconic acid to keep the fructose as sweetener (Cañete-Rodríguez et al., 2015). Additionally, alcoholic fermentation by *Saccharomyces cerevisiae*, is used to elaborate strawberry beverages (Hidalgo, Torija, Mas, & Mateo, 2013) The impact of gluconic fermentation has been evaluated in terms of amino acids and biogenic amines (Ordóñez et al., 2015). Besides, non-anthocyanin composition of these beverages (gluconic and alcoholic) has been described before showing their potential as a source of bioactive compounds (Álvarez-Fernández, Hornedo-Ortega, Cerezo, Troncoso, & García-Parrilla, 2014; Álvarez-Fernández, Cerezo, Cañete-Rodriguez, Troncoso, & García-Parrilla, 2015).

Among the healthy properties of strawberries, neuroprotective effects due to the anthocyanin content have been reported (Giampieri et al., 2015) Hence, recent studies show the effectiveness of anthocyanins against Aβ toxicity. Indeed, Badshah, Kim, & Kim (2015) demonstrated that an anthocyanin extract (cyanidin 3-glucoside, delphinidin 3-glucoside and petunidin 3-glucoside) of black soybean decreased the neuronal death in HT22 cells. Additionally, cyanidin 3-glucoside can inhibit $A\beta_{25-35}$ spontaneous aggregation into oligomers and their neurotoxicity in human neuronal SH-SY5Y cells (Tarozzi et al., 2010). To the best of our knowledge, the protective effect against Aβ peptide has not been explored neither with strawberry or its derivatives nor

for pelargonidin and derivatives compounds. Therefore, our work intends to explore the hypothetical activity these compound and beverages may present.

The aims of this paper are to (i) characterize the anthocyanin composition of fermented beverages elaborated from strawberry, (ii) to estimate the effect of alcoholic and gluconic fermentation on anthocyanin compounds, (ii) to evaluate their bioactive potential.

2. Material and methods.

2.1. Samples

Hudisa Desarrollo Industrial S.A. (Lepe, Spain) provided strawberry purée. The process of elaboration of the strawberry mash is summarized as follows: the fruit is received, selected, cleaned and the stems and leaves are eliminated. After the strawberry has been mashed, an inactivation enzymatic (2 min, 55 ºC–65 ºC) is performed, followed by a pasteurization process (3 min, >90 ºC). Finally, the temperature is reduced to 5 ºC. The mash is sieved to remove the seeds.

In this study, two harvests were analyzed (2012 and 2013). These purées were frozen (-20ºC) until fermentation was carried out. Alcoholic and gluconic fermentations were conducted in the Department of Inorganic Chemistry and Chemical engineering of the University of Córdoba (Córdoba, Spain). Fermentation conditions were previously described (Álvarez-Fernández et al., 2014, 2015). Alcoholic fermentation was carried out with a *Saccharomyces cerevisiae* (CET 13057 isolated from native strawberry yeast) used as a starter for the submerged fermentation process. The fermentation process was as follows: 3.6 L of strawberry purée were placed into the bioreactor and the conditions set (pH 3.32, 29 ºC, 26.20 rad.s^{-1}); the medium was saturated with oxygen

only at the beginning of the fermentation process, before adding the inoculum (10% (w/v) glucose, 0.1% (w/v) $MgSO_4$, 0.2% (w/v) KH_2PO_4, 0.3% (w/v) $(NH_4)_2SO_4$, 0.4% (w/v) yeast extract and 0.36% (w/v) bacteriological peptone). The end of the fermentation process was established when the glucose had been totally consumed and final pH was 3.30.

For gluconic fermentation, 3 L of strawberry purée substrate were placed into the bioreactor and the conditions set (pH 3.24, 29 ºC, 20% O_2 and 1250 g); after 10-20 min, 125 ml of inoculum of *Gluconobacter japonicus* strain E1 were added (5% (w/v) glucose, 1% (w/v) bacteria extract and 2% (w/v) bacteriological peptone) and mixed for 20-30 min, then the initial sample was taken. The end of the fermentation process was established when the glucose had been totally consumed and final pH was 2.74.

Eight alcoholic fermentation (code A) experiments were performed: four with purées from the 2012 harvest (code 12) and four with those from the 2013 harvest (code 13). Additionally, six gluconic fermentation experiments (code G) were performed: four with purées from the 2012 harvest and two from the 2013 harvest. Samples were taken at the initial point of the fermentation experiment (I), the final point (F) and after pasteurization of the fermented product (FP). Pasteurization was carried out at 70-80 ºC for 15 minutes. All samples were frozen until analysis. Table 1 displays the codes of the samples used in this study.

2.2. Chemicals and reagents

Amberlite XAD7HP, Dimethyl sulfoxide (DMSO), Dulbecco´s modified Eagle´s medium (DMEM)-Glutamax, Trypsine-EDTA, Thiazolyl Blue Tetrazolium Bromide (MTT), Phosphate Buffered Saline (PBS), L-glutamine, fetal horse serum and fetal bovine serum, streptomycin, 2',7'-dichlorofluorescin diacetate (DCFH-DA), 2,2-diphenyl-1-

picrylhydrazyl (DPPH), 2,2'-diazo-bis-amidinepropane-dihydrochloride (AAPH), and Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic) were purchased from Sigma (Steinheim, Germany). Fluorescein sodium was obtained through Fluka (Steinheim, Germany).

VWR Chemicals (Llinars del Vallés, Barcelona) supplied methanol and acetic acid. Formic acid and acetonitrile were obtained by Fisher Chemical. Pelargonidin 3-glucoside was obtained from Chloride (Cromadex Inc., USA). Cells PC12-Adh were supplied by ATCC® CRL-1721.1™ (Manassas, USA) and Amyloid β-protein 25–35 ($A\beta_{25-35}$) by Synvec (Bordeaux, France).

2.3. Sample preparation

An Amberlite XAD7HP column (30 x 1.5 cm) was conditioned with 200 mL of methanol and then 200 mL of water. A total of 20 g of sample (gluconic and alcoholic fermented samples) were diluted with water (1:1, w/v). The column was loaded with the diluted sample and cleaned with water to eliminate sugars, acids and polar compounds. Subsequently, the anthocyanin fraction was eluted with a mixture of methanol: acetic acid (19:1); flow rate 1 drop s^{-1}. This fraction was collected and concentrated with a rotary evaporator under vacuum (Büchi Rotavapor, R-200/205), frozen (-80ºC) and finally freeze-dried. A total of approximately 20 mg of this extract was obtained from 20 g of sample. The respective extracts were used for the subsequent analysis.

2.4. Analysis of anthocyanin compounds

Analyses were carried out using a UHPLC system model 1290 Infinity (Agilent, Palo Alto, CA), equipped with a binary pump (Agilent Technologies, 1290 VL G4220B), an

autosampler (Agilent Technologies, 1290 sampler, G4226A) and a DAD detector (Agilent Technologies, G1316C). The UHPLC system was coupled to a Bruker mass spectrometer (model Esquire 3000+) with electrospray ionization (ESI), Atmospheric Pressure Chemical Ionization (APCI) and an Ion Trap analyzer. Samples were filtered through a Millipore PTFE 0.45 µm filter before injection.

The sample volume injected was 1µL. A SB-C18 column (2.1 x 100 mm, 1.8 µm) (Agilent, USA) was used. Two different solvents were used as a mobile phase: solvent A (water/formic acid 90:10 v/v) and solvent B (acetonitrile/formic acid 90:10 v/v), at a flow rate of 0.4 ml min^{-1} and a gradient as follows: 0 min 1% B, 9 min 20% B, 13.44 min 100% B, 14.55 100% B and 14.77 1% B .

Data were processed using HyStar 3.2 software (Bruker). The MS/MS parameters were as follows: positive mode; capillary tension: -3700 V; nebulizer: 40psi; dry gas: 10 l min^{-1}; dry temperature: 365 °C; and scan range, *m/z*: 100 to 1200.

Anthocyanins in strawberry were quantified using the areas of their chromatographic peaks at 520 nm by comparison with a standard pelargonidin 3-glucoside calibration curve. Strawberry extracts were analyzed in duplicate.

2.5. Antioxidant activity

2.5.1. DPPH method

The DPPH free radical scavenging activity assay was performed according to Katalinic, Milos, Kulisic, & Jukic (2006) with slight modifications and using 96-well plates. A total of 50 µL of extracts (5-100 µg mL^{-1}) were added to each well and completed to a final volume of 200 µL with 150 µM DPPH methanolic solution. Plates were shaken for 10 s

and then absorbance was measured in a multi-detector microplate reader at 517 nm (Synergy HT, Biotek®). The inhibition percentage of DPPH radical (I%) was calculated as follows:

$$I\% = ((Abs_{DPPH} - Abs_S)/ Abs_{DPPH}) \times 100$$

Where Abs_{DPPH} is the absorbance of the control without sample and Abs_S is the absorbance of the tested sample measured at 20 min.

Sample concentration providing 50% inhibition (IC_{50}) was calculated from the graph, plotting inhibition percentage against sample concentration (µg mL^{-1}). All experiments were performed in triplicate.

2.5.2. ORAC test

The procedure is based on a previously reported method (Ou, Hampsch-Woodill, & Prior, 2001) with slight modifications: 50 µL of sample or Trolox are mixed with 100 µL of fluorescein (45 mM) and 50µL of AAPH (15 mM). Fluorescence was recorded for 90 min (excitation wavelength was set at 485 nm; emission wavelength at 528 nm). Measurements were taken in triplicate in a multi-detector microplate reader (Synergy HT, Biotek®). Trolox was used as a calibration standard at the 0.5-9.5 µM concentration range established previously (Cerezo, Cuevas, Winterhalter, García-Parrilla, & Troncoso, 2010).

Fluorescein fluorescence was recorded every 5 min after the addition of AAPH, until the fluorescence was <5% of the initial reading. The final results were calculated using the areas under the fluorescein decay curves between the blank and the sample and were expressed as µmols of Trolox g^{-1} of fresh weight.

221 2.6. Cell culture

222 PC12 cells derived from rat pheochromocytoma were cultured in a DMEM-Glutamax
223 medium containing 100 units mL^{-1} penicillin, 100 µg mL^{-1} streptomycin, 15% fetal horse
224 serum and 2.5% fetal bovine serum and maintained at 37 °C in a humidified incubator
225 with 5% CO_2.

226 PC12 were subcultured at a density of 30,000 cells per well, in 96-well culture plates in
227 200 µL of culture medium. After 24 h, cells were incubated with strawberry extracts
228 (10-100 µg mL^{-1}) or pelargonidin 3-glucoside (50-300 µM), in the presence or absence
229 of $A\beta_{25-35}$ (10 µM and 20 µM for viability and ROS measurement, respectively), in a
230 serum-free culture medium. Pelargonidin 3-glucoside was dissolved in DMSO at a final
231 concentration of 0.1% (v/v) and extracts were dissolved in water (1 mg mL^{-1}).

232 2.7. MTT reduction cell viability

233 After treatment (24 h), PC12 cells were incubated with 0.5 mg mL^{-1} of MTT for 3 h at
234 37 °C. The resulting crystals that formed were dissolved with 100 µL of DMSO and
235 were finally determined by measuring absorbance, using a microplate reader MRXII
236 (Dynex), at a wavelength of 540 nm. Data are expressed as means ± SEM.

237 2.8. Intracellular ROS measurement

238 Generation of ROS in cells was analysed using a fluorometric probe (DCFH-DA). After
239 treatment (6 h), cells were washed with PBS, and 5 µM of DCFH-DA was added. The
240 fluorescence intensity was immediately quantified for 30 min at 37 °C using a
241 spectrofluorometer (FLUOstar Optima, BMG Labtech). The wavelengths of excitation
242 and emission used to detect the ROS were 485 nm and 520 nm, respectively. All

243 experiments were performed in the dark. Results were given as the percentage of ROS
244 production compared with the untreated control cells.

245 2.9. Statistical analysis

246 One-way analysis of variance (ANOVA test) ($p < 0.05$) was used to explore significant
247 differences in anthocyanin composition before and after the fermentation and
248 pasteurization processes, using statistical software (Statsoft, 2004). The IC_{50} was
249 calculated using Graphpad Prism 6 software. For ROS and cell viability measurements,
250 the statistical tests were performed with one-way ANOVA (statistical software),
251 followed by Dunnett's multiple comparison post-hoc test. Significance was set at $p <$
252 0.05.

253

254 **3. Results and discussion**

255 3.1. Anthocyanin characterization

256 The chromatogram profile of the strawberry samples is presented in Figure 1. A total
257 of eleven compounds were identified in both alcoholic and gluconic fermented
258 products by LC-DAD-MS/MS. Table 2 summarizes the retention time and mass
259 spectrometry characteristics of those compounds compared with data reported in the
260 literature.

261 Pelargonidin 3-glucoside (peak 7) was the major compound, followed by pelargonidin
262 3-rutinoside (peak 8) and cyanidin 3-glucoside (peak 5), in accordance with previously
263 reported data (Pérez-Jímenez et al., 2010; García-Viguera et al., 1998).

264 Catechin-(4-8)-pelargonidin 3-glucoside and epicatechin-(4-8)-pelargonidin 3-glucoside
265 (peaks 1 and 2, respectively) were also identified. They both presented identical
266 molecular ions at *m/z* 721 and fragmentation patterns (559/407/313), but had
267 different retention times. Additionally, other anthocyanin linked flavonols, such as
268 (epi) afzelechin-pelargodinin 3-glucoside (peak 4) were identified. We also detected
269 delphinidin 3-glucoside (peak 3) and 5-carboxypyranopelargonidin 3-glucoside (peak 9)
270 in these samples, which were previously reported for the first time by our group in
271 *Camarosa* strawberry purée (Cerezo et al., 2010). Furthermore, other pelargonidin
272 derived compounds were identified as follows: peak 10 showed a molecular ion at *m/z*
273 519 and a fragmentation pattern at *m/z* 271 corresponding to pelargonidin 3-(6''-
274 malonylglucoside); peak 11 was consistent with pelargonidin 3-acetylglucoside,
275 showing a molecular ion at *m/z* 475 and fragmentation pattern at *m/z* 271; and peak 6
276 corresponded to pelargonidin 3,5-diglucoside (*m/z* 595/433/271), all of them in
277 accordance with previous reports (Lopes-da-silva et al., 2002, 2007).

278 3.2. Effects of alcoholic and gluconic fermentation processes

279 Table 3 displays anthocyanin composition data after alcoholic fermentation. Two of
280 the major compounds cyanidin 3-glucoside and pelargonin 3-glucoside decreased
281 significantly ($p<0.05$) after the fermentation process (51.5 to 38.3% and 56.3 to 38.7%
282 respectively, considering both harvests). Other authors have also reported a reduction
283 in total anthocyanins (85-63%) in strawberry wine as a result of the alcoholic
284 fermentation process. (Klopoyek, Otto, & Böhm, 2005; Ubeda et al., 2013). Our results
285 reveal that the total anthocyanin decrease is due specifically to cyanidin 3-glucoside
286 and pelargonidin 3-glucoside. In contrast, pelargonidin 3,5-diglucoside significantly

287 ($p<0.05$) increased its concentration after the fermentation process, in both harvests
288 (2.5 and 6.2 times higher, respectively).

289 On the other hand, gluconic fermentation preserved all the anthocyanin compounds,
290 since no significant changes took place after this process (Table 4). Different factors
291 could explain the observed results. Firstly, the decrease in the pH value obtained after
292 gluconic fermentation (from an initial 3.24 to 2.74) while alcoholic fermentation does
293 not change significatively (from 3.32 to 3.30). The acidic pH obtained after the
294 conversion of glucose into gluconic acid by *G. japonicus* probably stabilizes these
295 compounds. On the other hand, an adsorption mechanism between the yeast (*S.*
296 *cerevisiae*) and anthocyanin compounds has been stated as responsible of the
297 decrease on anthocyanin in alcoholic fermented final products (Morata et al., 2003).
298 Our work reveals that *G. japonicus* does not interact with anthocyanin compounds in
299 such an extent that could make a significant difference in the beverage.

300 Gluconic fermentation is therefore an advantageous process for maintaining
301 anthocyanin compounds, as has also been shown for non-anthocyanin phenolic
302 compounds in the gluconic fermentation of strawberry (Álvarez-Fernández et al, 2014).

303 ### 3.3. Antioxidant activity, DPPH and ORAC method

304 Table 5 shows antioxidant activity expressed in Half Maximal Inhibitory Concentration
305 (IC_{50}) (DPPH method) and µmols of Trolox g^{-1} of fresh weight (ORAC method). The
306 results of the ORAC assays showed similar values in all cases (I, F and P). Antioxidant
307 activity is conserved in all extracts, regardless the process involved. Furthermore,
308 DPPH assays show the same tendency and it can be concluded that gluconic and
309 alcoholic fermentation leave *in vitro* antioxidant activity practically unchanged.

3.4. Cell viability of PC12 and ROS production

To evaluate the protective effects of strawberry fermented extracts, their capacity to protect rat pheochromocytoma derived PC12 cells from Aβ peptide induced toxicity was measured. An MTT assay was used to determine cell viability. Three extracts (I, and A and G final samples, 2013 harvest) were selected in order to identify the impact of alcoholic and gluconic fermentation on the activity of the extract. In particular, those extracts with higher concentration in cyanidin 3-glucoside, pelargonidin 3-glucoside, pelargonidin 3-rutinoside and pelargonidin 3-(6''-malonylglucoside) according to the values displayed in Tables 3 and 4 were used for further experiments.

First, the cytotoxic potential of the extracts at different concentrations ranging from 10 to 200 μg mL^{-1} on PC12 cells was measured. We observed a toxic effect for PC12 cells at 200 μg mL^{-1} in all tested extracts (data not given). Figure 2 displays cell viability expressed as a percentage relative to the untreated control cells. After exposure to $A\beta_{25-35}$ alone, viability decreased by more than 60%, compared with the control. However, when cells were treated with $A\beta_{25-35}$ in the presence of the strawberry extracts, cell viability increased significantly ($p<0.05$) (16 to 57%), in a dose-dependent manner. Specifically, 100 μg mL^{-1} of gluconic extract reversed amyloid-induced toxicity and increased cell viability up to near the control level (Figure 2). Other authors have reported that acai berry extracts (50 μg mL^{-1}) did not provide any significant degree of protection against $A\beta_{25-35}$-mediated loss of cell viability PC12; however, they had an effect when tested with $A\beta_{1-42}$ at 5 μM, with 25 and 29% increased cell viability at 5 and 50 μg mL^{-1}, respectively (Wond, Musgrave, Harvey, & Smid, 2013). Additionally, blueberry leaf extracts (50-100 μg mL^{-1}) caused a small cell viability increase of 6-15%

on $A\beta_{25-35}$-induced cytotoxicity in PC12 cells (Jeong et al., 2013). Harvey, Musgrave, Ohlsson, Franson, & Smid (2011) found that grape-skin extract tested at different concentrations of $A\beta_{1-42}$ provided protection against amyloid toxicity (37%) in PC12 cells. As far as we know, the strawberry extract studied in this paper shows one of the highest protective effects against Aβ-induced damage.

The effect of the extracts on cell viability could be explained as being due to the presence of pelargonidin 3-glucoside, which is the major compound in our extracts. For this reason, we evaluated the cytoprotective ability of pelargonidin 3-glucoside standard on PC12 cells. Cell viability was decreased after cells were exposed to 300 µM (data not shown). However, a protective effect was determined at concentrations of 50, 100 and 200 µM (21.6, 43.2 and 86.4 µg mL^{-1}, respectively), in a dose-dependent manner (Figure 2B). In gluconic fermented extract (G13F) at the highest concentration tested (100 µg mL^{-1}), pelargonidin 3-glucoside concentration is 12.9 µg mL^{-1}. Since the pelargonidin 3-glucoside at concentration 21.6 µg mL^{-1} (= 50 µM) increased cell viability by just 10%, the protective effects of gluconic fermented extract are not only due to the pelargonidin 3-glucoside. Other anthocyanins described here and polyphenols previously identified in strawberry may contribute to the protective activity in our extracts (Cerezo et al., 2010). Indeed, other authors have reported that cyanidin 3-glucoside and ellagic acid also contribute to the neuroprotective effect against $A\beta_{25-35}$ and $A\beta_{1-42,}$ respectively, in SH-SY5Y cells (Tarozzi et al., 2010; Feng et al., 2009).

It has been postulated that ROS are important players in degenerative diseases (Emerit, Edeas & Bricaire, 2004). ROS generate protein and lipid oxidations, DNA

damage and induce the death of neuronal cells (Wang et al., 2014). The neurotoxic effect of Aβ is associated with the production of ROS. Recent studies showed that cyanidin and malvidin 3-glucoside have protective effects against Aβ-induced neurotoxicity through inhibiting ROS formation (Thummayot et al., 2014; Shih, Wu, Yeh, & Yen, 2011)

In this study, we evaluated the effect of G13F and A13F extracts on ROS production by measuring intracellular ROS levels with the DCFH-DA assay. As shown in Figure 3, $A\beta_{25-35}$ treatment for 6 h induced a 1.8- to 2.4-fold increase in ROS production. Co-treatment with different concentrations of strawberry extracts significantly attenuated intracellular ROS accumulation. Strawberry extracts decreased the production of ROS in a dose-dependent manner that can attenuate Aβ-induced oxidative stress proving this specific antioxidant activity.

4. Conclusion

Alcoholic fermentation of strawberry pureés decreased the anthocyanin content while gluconic fermentation preserved these compounds, which is an advantage of this last process. All these extracts were protective against Aβ-peptide neurotoxicity in PC12 cells, with gluconic fermented strawberry extracts being the most effective, as determined by cell viability and intracellular ROS production. Taken together, our experiments suggest that the fermented derivatives of strawberry are a good source of antioxidant bioactives.

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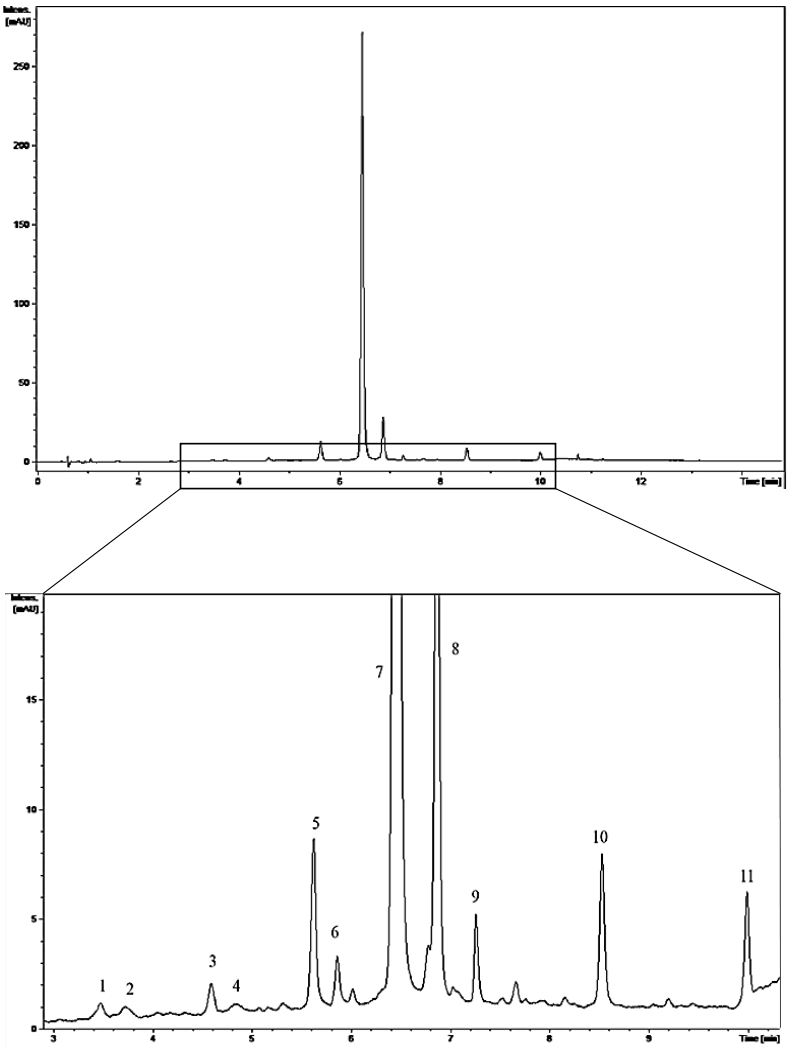
494

495

Figure 1.



Chromatogram and chromatogram (zoom) recorded at 520 nm showing the anthocyanin profile of the strawberry samples. 1: Catechin-(4-8)-pelargonidin 3-glucoside; 2: Epicatechin-(4-8)-Pelargonidin 3-glucoside; 3: Delphinidin 3-glucoside; 4: (epi)Afzelechin-pelargodinin 3-glucoside; 5: Cyanidin 3-glucoside; 6: Pelargonidin 3,5-diglucoside; 7: Pelargonidin 3-glucoside; 8: Pelargonidin 3-rutinoside; 9: 5-

150.

carboxypyranopelargonidin 3-glucoside; 10: Pelargonidin 3-(6''-malonylglucoside); 11: Pelargonidin 3-acetylglucoside.

Figure 2A and 2B.

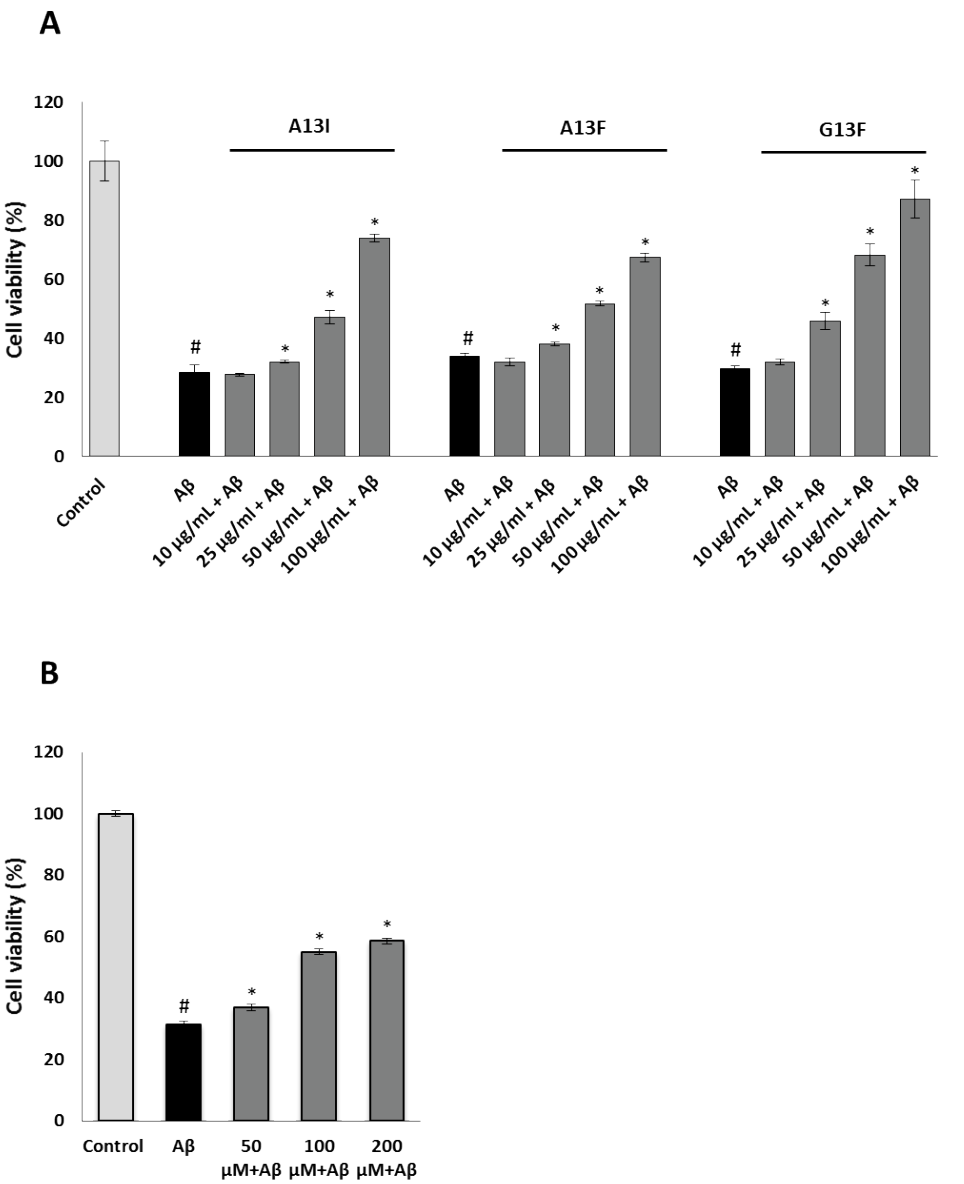


Figure 2 (A-B). Cells viability determination. Cells were treated 24h by strawberry extracts (A) or pelargonidin 3-glucoside (B), in presence or absence of 10 µM of $A\beta_{25\text{-}35}$.

Results are expressed as mean SEM of four replicates (n=4). $^{\sharp}$ P < 0.05 $A\beta_{(25-35)}$ versus control, * P < 0.05 extract versus $A\beta_{(25-35)}$.

Figure 3.

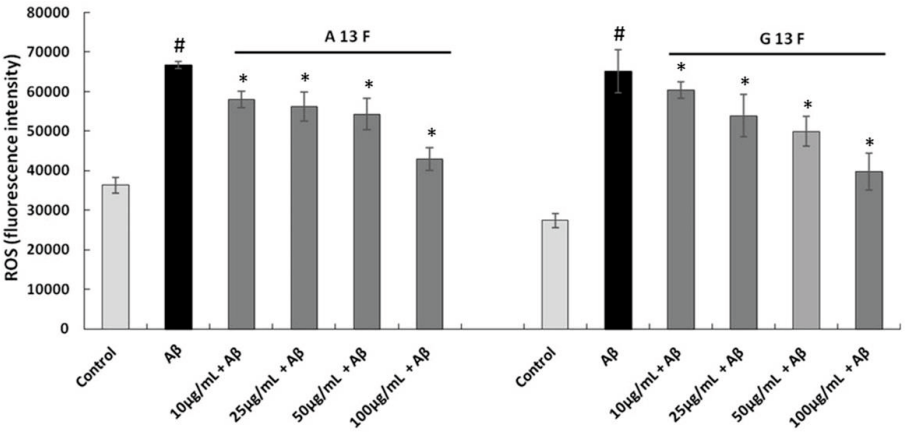


Figure 3. ROS (Fluorescence intensity). Production of ROS in PC12. Cells were treated for 6 h by alcoholic and gluconic strawberry extracts, in presence or absence of 20 µM of Aβ $_{(25-35)}$. Results are expressed as mean SEM of four replicates (n=4). ♯ $P < 0.05$ Aβ $_{(25-35)}$ versus control, * $P < 0.05$ extract versus Aβ $_{(25-35)}$.

Table 1. Sample codes

Codes	Sample
A 12 I	Alcoholic 2012 Harvest Initial
A 12 F	Alcoholic 2012 Harvest Final
A 12 FP	Alcoholic 2012 Harvest Pasteurized
A 13I	Alcoholic 2013 Harvest Initial
A 13 F	Alcoholic 2013 Harvest Final
A 13 FP	Alcoholic 2013 Harvest Pasteurized
G 12 I	Gluconic 2012 Harvest Initial
G 12 F	Gluconic 2012 Harvest Final
G 12 FP	Gluconic 2012 Harvest Pasteurized
G 13 I	Gluconic 2013 Harvest Initial
G 13 F	Gluconic 2013 Harvest Final
G 13 FP	Gluconic 2013 Harvest Pasteurized

Table 2. Identification of anthocyanin compounds of alcoholic- and gluconic-fermented products of strawberry purée.

*T_R, retention time.

Peak	T_{R*} (min)	Molecular ion $[M^+]$ (*m/z*)	MS^2 *(m/z)*	Identification	References
1	3.5	721	559/407/313	Catechin-(4,8)-pelargonidin 3-glucoside	Lopes-da-Silva et al., 2007 ; Fossen, Rayyan, & Andersen, 2004
2	3.7	721	559/407/313	Epicatechin-(4,8)-pelargonidin 3-glucoside	Lopes-da-Silva et al., 2007
3	4.4	465	303	Delphinidin 3-glucoside	Cerezo et al., 2010
4	4.6	705	545/407/313	(epi)Afzelechin-pelargonidin 3-glucoside	Lopes-da-Silva et al., 2007
5	5.6	449	287	Cyanidin 3-glucoside	Wu, & Prior, 2005; Lopes-da-Silva, 2002
6	6.0	595	433/271	Pelargonidin 3,5-diglucoside	Lopes-da-Silva et al., 2007; Cerezo et al., 2010 ; Wu, & Prior, 2005
7	6.5	433	271	Pelargonidin 3-glucoside	Wu, & Prior, 2005 ; Lopes-da-Silva, 2002
8	6.8	579	433/271	Pelargonidin 3-rutinoside	Wu, & Prior, 2005 ; Lopes-da-Silva, 2002
9	7.3	501	339	5-carboxypyranopelargonidin 3-glucoside	Cerezo et al., 2010
10	8.6	519	271	Pelargonidin 3-(6''-malonylglucoside)	Wu, & Prior, 2005
11	9.9	475	271	Pelargonidin 3-acetylglucoside	Lopes-da-silva et al., 2007; Wu, & Prior, 2005

Table 3. Concentration (mg kg^{-1}) of alcoholic-fermented extracts.

Compounds	A12I	A12F	A12FP	A13I	A13F	A13FP
Delphinidin 3-glucoside	0.64 ± 0.15^{ab}	0.30 ± 0.10^{a}	0.36 ± 0.16^{b}	0.69 ± 0.13^{b}	0.52 ± 0.22	0.41 ± 0.15^{b}
Cyanidin 3-glucoside	3.30 ± 0.80^{ab}	1.60 ± 0.40^{a}	1.90 ± 0.40^{b}	4.70 ± 0.60^{ab}	2.90 ± 0.50^{a}	2.70 ± 0.30^{b}
Pelargonidin 3,5-diglucoside	0.10 ± 0.07^{ab}	0.25 ± 0.10^{a}	0.21 ± 0.11^{b}	0.13 ± 0.04^{ab}	0.81 ± 0.13^{ac}	0.65 ± 0.04^{bc}
Pelargonidin 3-glucoside	61.20 ± 13.08^{ab}	26.7 ± 7.8^{ac}	34.90 ± 5.40^{bc}	116.2 ± 13.3^{ab}	71.72 ± 10.24^{a}	66.07 ± 8.13^{b}
Pelargonidin 3-rutinoside	10.35 ± 2.02^{ab}	7.80 ± 1.80^{a}	8.11 ± 1.14^{b}	11.90 ± 1.60^{b}	11.90 ± 1.80^{c}	9.90 ± 1.60^{bc}
5-carboxypyranopelargonidin 3-glucoside	0.73 ± 0.11	0.67 ± 0.09	0.68 ± 0.15	0.97 ± 0.12	1.06 ± 0.16	0.94 ± 0.18
Pelargonidin 3-(6”-malonylglucoside)	1.31 ± 0.22^{ab}	0.88 ± 0.20^{a}	0.99 ± 0.14^{b}	3.00 ± 0.40^{b}	2.80 ± 0.40^{c}	2.46 ± 0.23^{bc}
Pelargonidin 3-acetylglucoside	0.65 ± 0.15^{a}	0.48 ± 0.13^{a}	0.52 ± 0.10	1.80 ± 0.30^{b}	1.72 ± 0.23	1.50 ± 0.24^{b}

[a] Superscript letter indicates significant difference ($p < 0.05$) between Initial (I) and Final point (F) and Final Pasteurized point (FP) within the same harvest.
[b] Superscript letter indicates significant difference ($p < 0.05$) between Initial (I) and Final point Pasteurized (F) and Final Pasteurized point (FP) within the same harvest.
[c] Superscript letter indicates significant difference ($p < 0.05$) between Final point (F) and Final point Pasteurized (F) and Final Pasteurized point (FP) within the same harvest.
Sample identification in Table 1

Table 4. Concentration (mg kg^{-1}) of gluconic-fermented extracts

Compounds	G12I	G12F	G12FP	G13I	G13F	G12FP
Delphinidin 3-glucoside	0.46 ± 0.10	0.35 ± 0.20	0.33 ± 0.22	0.41 ± 0.04[b]	0.60 ± 0.40	0.85 ± 0.03[b]
Cyanidin 3-glucoside	2.80 ± 0.30[b]	2.30 ± 0.70	2.10 ± 0.50[b]	4.79 ± 0.54	5.20 ± 0.50	5.26 ± 0.22
Pelargonidin 3,5-diglucoside	0.09 ± 0.03	0.10 ± 0.05	0.06 ± 0.01	0.12 ±0.01	0.14 ± 0.03	0.15 ± 0.00
Pelargonidin 3-glucoside	51.77 ± 6.06[b]	44.78 ±12.24	41.60 ± 9.80[b]	122.19 ± 11.22	129.1 ± 11.5	131.91 ± 5.18
Pelargonidin 3-rutinoside	8.07 ± 1.24	7.30 ± 2.30	6.60 ± 1.70	12.20 ±0.40[a]	13.60 ± 0.30[a]	13.4 ± 0.9
5-carboxypyranopelargonidin 3-glucoside	0.57 ± 0.12[b]	0.61 ± 0.12	0.68 ± 0.08[b]	0.50 ± 0.03[ab]	1.12 ± 0.15[a]	1.15 ± 0.12[b]
Pelargonidin 3-(6''-malonylglucoside)	0.99 ± 0.15	0.90 ±0.16	0.91 ± 0.04	3.60 ±0.40	3.26 ± 0.20	3.25 ± 0.17
Pelargonidin 3-acetylglucoside	0.53 ± 0.09	0.48 ± 0.14	0.46 ± 0.12	1.86 ± 0.22	1.95 ± 0.12	1.97 ± 0.05

[a] Superscript letter indicates significant difference ($p < 0.05$) between Initial (I) and Final point (F) and Final Pasteurized point (FP) within the same harvest.
[b] Superscript letter indicates significant difference ($p < 0.05$) between Initial (I) and Final point Pasteurized (F) and Final Pasteurized point (FP) within the same harvest.
[c] Superscript letter indicates significant difference ($p < 0.05$) between Final point (F) and Final point Pasteurized (F) and Final Pasteurized point (FP) within the same harvest.
Sample identification in Table 1

Table 5. Antioxidant activity for DPPH ($IC_{50:}$ Half Maximal Inhibitory Concentration) and ORAC (µmols Trolox g^{-1} fresh weight) methods.

	DPPH		ORAC
Samples	IC_{50} (µg mL^{-1})	CI (Confidence Interval)	(µmols Trolox g^{-1} fresh weight)
A12I	16.32	14.81-17.97	10.4 ± 2.3
A12F	17.57	16.30-19.93	12.8 ± 2.4
A12FP	19.99	17.06-23.42	8.5 ± 1.8
A13I	15.88	14.16-17.82	19.1 ± 1.0
A13F	15.55	14.17-17.06	14.8 ± 1.2
A13FP	18.20	15.39-21-53	15.1 ± 0.8
	DPPH		ORAC
Samples	IC_{50}	CI (Confidence Interval)	(µmols Trolox g^{-1} fresh weight)
G12I	17.93	15.36-20.94	9.4 ± 1.7
G12F	20.18	18.86-21.59	12.2 ± 1.1
G12FP	19.31	15.46-24.11	7.6 ± 2.9
G13I	18.26	13.90-24.00	20.7 ± 2.7
G13F	14.86	13.19-16.74	13.9 ± 0.8
G13FP	17.04	14.07-20.65	12.1 ± 1.5

Sample identification in Table 1

Capítulo 3

Estabilidad e influencia de las condiciones de almacenamiento en el perfil antociánico y color de una novedosa bebida obtenida por fermentación glucónica de fresa

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Influence of storage conditions on the anthocyanin profile and colour of an innovative beverage elaborated by gluconic fermentation of strawberry.

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Influence of storage conditions on the anthocyanin profile and colour of an innovative beverage elaborated by gluconic fermentation of strawberry.

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Abstract

Derived fruit products such as strawberry-based fermented beverages, increase the fruit's conservation period and offer new alternatives for the non-alcoholic market. The influence of storage time and temperature on the anthocyanin composition, antioxidant activity and colour of a fermented strawberry beverage were studied and indicated 60-days is the half-life for the tested beverage. Twenty-three anthocyanin compounds were analysed by Ultra High-Performance Liquid Cromatography Orbitrap (UHPLC-MS/MS). This is the first time that pelargonidin 3-sambubioside, delphinidin 3-arabinoside, cyanidin 3-(6-acetyl)-glucoside and delphinidin 3-galactoside have been reported in any products derived from strawberry. Additionally, the accurate mass of the following anthocyanins were reported: catechin-(4-8)-pelargonidin 3-glucoside, afzelechin-pelargonidin 3-glucoside, pelargonidin dissacharide-(hexose+pentose)-acylated with acetic acid, 5-carboxypyranopelargonidin 3-glucoside, pelargonidin 3-arabinoside, pelargonidin 3-malonylglucoside, pelargonidin 3-(6-acetyl)-glucoside and pelargonidin 3-(6-succynil)-arabinoside. Functional aspects of this drink rely on its bioactive compounds and lack of glucose due to its transformation to gluconic acid which makes it suitable for diabetic consumers.

Keywords: fermented products, drink, strawberry, bioactive compounds, gluconic, storage.

Pelargonidin 3-glucoside (PubChem CID: 443448); Pelargonidin 3-sambubioside (PubChem CID: 71627264); Pelargonidin 3-rutinoside (PubChem CID: 44256626); Pelargonidin 3-arabinoside (PubChem CID: 44256694); Pelargonidin 3- (6"-malonylglucoside) (PubChem CID: 45256635); Cyanidin 3-galactoside (PubChem CID:

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44256700); Cyanidin 3-glucoside (PubChem CID: 92131208); Peonidin 3-glucoside (PubChem CID: 443654); Delphinidin 3-arabinoside (PubChem CID: 12137508); Delphinidin 3-glucoside (PubChem CID: 443650).

Abbreviations:

UHPLC-MS/MS, Ultra High-Performance Liquid Chromatography; eNOS, endothelial Nitric Oxide Synthase; NO, Nitric Oxide; ORAC, Oxygen Radical Absorbance Capacity; DPPH, 2,2-diphenyl-1-picrylhydrazyl

1. Introduction

Strawberry [*Fragaria x ananassa* (*Rosaceae* Family)] is harvested in a very short period of time and is a very perishable product leading to the fruit rotting. The fruit has to be discarded if not sold, which entails substantial economic loss. Consequently, manufacturing derived products, such as fermented beverages using strawberries as a raw material, increases the conservation period and offers new sustainable and successful alternatives for the strawberry market, which drive economic profit.

Strawberry is a good source of nutrients, phytochemicals and fibre. The anthocyanins are among the principal bioactive compounds in strawberry (Basu, Nguyen, Betts, & Lyons, 2014). Additionally, these compounds are crucial for its colour quality. Several investigations have revealed antioxidant, anti-inflammatory, antihypertensive and antihyperlipidemic or antiproliferative effects of strawberry anthocyanin compounds (Basu et al., 2014). Reduction on the risk of hypertension (8%) was observed when the consumption of strawberry anthocyanin was between 16 and22 mg/day compared with a lower consumption (5-7 mg/day of anthocyanins) (Cassidy et al., 2011). Pure cyanidin 3-glucoside, induced endothelial nitric oxide synthase (eNOS) expression and increased nitric oxide (NO) release, which may help ameliorating endothelial dysfunction and maintain the blood pressure (Xu, Ikeda, & Yamori, 2004). Antioxidant and antiproliferative activities *in vitro* have been reported by Zhang, Seeram, Lee, Feng, and Heber (2008) when evaluating purified anthocyanin compounds in different human cancer cells (oral, colon and prostate).

It is well known that pelargonidin 3-glucoside is the major anthocyanin in strawberry (153–652 mg/kg fresh weight) followed by pelargonidin 3-rutinoside and other

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pelargonidin and cyanidin derivatives (Lopes-da-Silva, Escribano-Bailon, Perez-Alonso, Rivas-Gonzalo & Santos-Buelga, 2007; Cerezo, Cuevas, Winterhalter, Garcia-Parrilla, & Troncoso, 2010a).

Recently, delphinidin 3-glucoside, peonidin 3-glucoside, and cyanidin 3-galactoside were identified in strawberry for the first time (Cerezo et al., 2010a). Other minor anthocyanin compounds, such as 5-carboxypyranopelargonidin 3-glucoside, pelargonidin acylated derivatives and pelargonidin linked to flavanol, were also present in strawberry (Lopes-da-Silva, de Pascual-Teresa, Rivas-Gonzalo & Santos-Buelga, 2002; Lopes-da-Silva et al., 2007; Fossen, Rayyan, & Andersen, 2004).

Different types of processes are used to obtain more appropriate and attractive strawberry derivative products. The anthocyanin composition of processed products from berries such as liqueurs, juices, nectar, purée, condiments and jams has been studied only in terms of total anthocyanins (Sokól-Lętowska et al., 2014; Da Silva, Lajolo & Genovese, 2007; Klopoyek, Otto, & Böhm, 2005; Ubeda, Callejón, Hidalgo, Torija, Troncoso, & Morales, 2013). However, there are no previous studies involving characterization of anthocyanin profile of strawberry fermented products, being restricted to non-anthocyanin composition (Álvarez-Fernández, Hornedo-Ortega, Cerezo, Troncoso, & García-Parrilla, 2014).

Process, time, and storage temperature are crucial factors that influence significantly the stability of anthocyanin compounds (Clifford, 2000). Tiwari, O´Donnel, Patras, Brunton and Cullen (2009) observed greater stability of pelargonidin 3-glucoside and antioxidant activity at 4 ºC compared to 20 ºC in strawberry juice. Furthermore, anthocyanin compounds in liqueurs made from red fruits have been preserved for 3

months at 15 ºC (Sokól-Lętowska et al., 2014). Therefore, the stability of anthocyanin compounds in each food matrix might be different. Indeed alcoholic and acetic fermentation exert a different impact on anthocyanin profile. Effects of alcoholic fermentation have been extensively studied. During red wine fermentation, the monomeric anthocyanins undergo several reactions and associations leading to the formation of anthocyanin-derived pigments. These reactions include self-association and copigmentation, such as the formation of polymeric anthocyanins with flavan-3-ols and proanthocyanidins, as well as the formation of new pigments, such as pyranoanthocyanins (Wrolstad, Durst, & Lee, 2005; Jackson, 2008; Brouillard, Chassaing, & Fougerousse, 2003). Acetification process of red wine also modifies anthocyanin composition, increasing vitisin-type and ethyl-linked compounds and decreasing the monomeric anthocyanins (Cerezo, Cuevas, Winterhalter, Garcia-Parrilla, & Troncoso, 2010b). However, the impact to anthocyanin composition of the gluconic fermented matrix products has not been studied.

These beverages represent an innovative trend as they lack glucose and therefore can be consumed by diabetics who can benefit from the health effects of anthocyanin.

The aim of this study was to examine the effect of storage time and temperature on the bioactive compounds (anthocyanins), antioxidant activity and colour as this sensory parameters influence consumer acceptability of a strawberry beverage obtained through gluconic fermentation UHPLC coupled with a hybrid mass spectrometer, which combined a linear trap quadrupole (LQT) and an Orbitrap mass analyzer, was used to identify and quantify the anthocyanin compounds. Finally, the antioxidant activity of the extracts was evaluated using oxygen radical absorbance

capacity (ORAC) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) essays; their colour parameters were also studied.

2. Materials and methods

2.1. Chemicals and materials

Amberlite XAD7HP, DPPH, 2,2´-diazo-bis-amidinepropane-dihydrochloride (AAPH), and Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic) were purchased from Sigma (Steinheim, Germany). Fluorescein sodium was obtained by Fluka (Steinheim, Germany). Acetonitrile was obtained from Merck (Darmstadt, Germany) and formic acid was obtained from Panreac (Barcelona, Spain).

Pelargonidin 3-glucoside, cyanidin 3-glucoside, delphinidin 3-glucoside and peonidin 3-glucoside were purchased from Chromadex Inc. (Irvine, California USA).

2.2. Gluconic fermented strawberry beverage

The strawberry beverage was obtained by gluconic fermentation previously described (Álvarez-Fernández et al., 2014). The process is detailed below: strawberry purées were the substrate for fermentation and provided by Hudisa Company (Lepe, Huelva, Spain). *Gluconobacter japonicus* was used as a starter for the fermentation process. The fermentation was carried out by submerged culture. Three litres of strawberry purée substrate were placed into the bioreactor and the conditions set (29 °C, 20% O_2 and 1250 g); after 10–20 min, 125 mL of inoculum of *G. japonicus* strain E1 were added and mixed for 20–30min. The process starts as the strawberry purée is mixed with the inoculum (strawberry substrate before fermentation) and finishes as the remaining glucose is totally consumed, then the fermentor is discharged. Subsequently, this product is immediately pasteurized by heating at 70–80 °C for 15 min and then rapidly

cooled and frozen. These fermented strawberry products were supplied by the Department of Analytical Chemistry, Chemical Engineering, Faculty of Sciences, University of Cordoba (Cordoba, Spain)

Finally, the beverage (Samples 0) was formulated according to the following procedure. The fermented product obtained as describe before was centrifuged (1500 g) and the supernatant was collected and mixed with carbonated water (80:20, v/v). A few gouts of Stevia® were added to increase sweetness to the final product. We divided this product into different aliquots for the purpose of this study. Aliquots were stored at different temperatures (4 and 25ºC) and samples were taken at different times (0, 15, 30, 60 and 90 days). Sample codes indicate: initial (0), samples at room temperature at 15, 30, 60 and 90 days (R15, R30, R60 and R90) and samples at fridge temperature at 15, 30, 60 and 90 days (F15, F30, F60 and F90).

2.3. Sample preparation

The method was carried out according Cerezo et al. (2010a). An Amberlite XAD7HP column (30 x 1.5 cm) was conditioned with 200 mL of methanol and then 200 mL of water. Subsequently, a total of 20-30 mL of sample were loaded onto the column and cleaned with water to eliminate sugars, acids and polar compounds. Later, the anthocyanin fraction was eluted with a mixture of methanol/acetic acid (19:1, v/v); flow rate 1 drop/s. This fraction was collected and concentrated with a rotary evaporator under vacuum (Büchi Rotavapor, Flawil, Switzerland). The extracts were reconstituted in 2 mL of acidified water (5% formic acid) and stored at -20 ºC until analysis.

2.4. UHPLC-MS/MS Orbitrap analysis

All analyses were performed using a liquid chromatography system consisting of a quaternary Rs Pump Dionex Ultimate 3000 (serial number: 8077352) and Rs autosampler Dionex Ultimate 3000 (serial number 8077399), connected to a quadrupole-orbitral (Qexactive) hybrid mass spectrometer with heated-electrospray ionization probe (HESI-II, Thermo Fisher Scientific, Bremen, Germany). Xcalibur software (version 3.0.63) was used for instrument control, data acquisition and data analysis. The analytical method was previously published (Natic et al., 2015). Separation was performed on a column SB-C18 (2.1 x 100 mm, 1.8 µm) (Agilent, USA). Injection volume was 1 µL and flow rate was 0.4 mL/min. Two different solvents were used as a mobile phase: Solvent A (Water/formic acid 95:5, v/v) and solvent B (acetonitrile/formic acid 95:5, v/v), scheduled in the following gradient: 0.0-2.0 min 5% B, 2.0-12.0 min from 5to 100 % B, 12.0-13.0 min from 100 to 5% B, then 5% B up to 15.0 min. Anthocyanin identification and quantification were acquired in positive mode by full-range acquisition covering *m/z* 100-1500 at 35,000 resolution and by targeted MS^2 normalized Higher Energy Collision Dissociation (HCD). Compounds were identified according to their mass spectra, calculated mass, characteristic fragmentation and retention time. Above mentioned available standards were used both for identification and quantification purposes. HESI source parameters were as follows: cell at 20 eV, source voltage 3.5 kV tube lens voltage 50 V, capillary temperature 263 ºC, and sheath and auxiliary gas flow rate (N_2) 50 and 13 (arbitrary units).

2.5. **Antioxidant activity**

2.5.1. DPPH method

Scavenging activity against the stable colored DPPH free-radical was assayed according to Katalinic, Milos, Kulisic, and Jukic (2006) with slight modifications, using 96-well plates. A total of 50 µL of extracts (1-15 µL/mL) were added to each well and completed to a final volume of 200 µL with 150 µM of methanolic solution of DPPH. Plates were shaken for 10 s and then absorbance was read after 20 min in a multi-detector microplate reader at 517 nm (Synergy HT, Biotek®, Winooski, Vermont, USA). The inhibition percentage of DPPH radical (I%) was calculated as follows:

$$I\% = ((A_C - A_S)/ As) \times 100. \quad (1)$$

Where A_C is absorbance of the control without sample and A_S is absorbance of the tested sample measured at 20 min.

Sample concentration providing 50% inhibition (IC_{50}) was calculated from the graph, plotting inhibition percentage against sample concentration (µL/mL). All experiments were performed in triplicate

2.5.2. ORAC test

The procedure is based on a previously reported method (Ou, Hampsch-Woodill, & Prior, 2001) with slight modifications. Fifty microlitres of sample or Trolox were mixed with 100 µL of fluorescein (45 mM) and 50 µL of AAPH (15 mM). Fluorescence was recorded for 90 min (excitation wavelength was set at 485 nm and emission wavelength at 528 nm). Measurements were taken in triplicate in a multi-detector

microplate reader (Synergy HT, Biotek®). Trolox was used as a calibration standard in the 0.5-9.5 µM concentration range previously established (Cerezo et al., 2010a).

Fluorescein fluorescence was recorded every 5 min after addition of AAPH until fluorescence was <5% of the initial reading. Final results were calculated using the areas under the fluorescein decay curves between the blank and the sample and were expressed as millimol Trolox equivalents/mL beverage.

2.6. Determination of colour stability

Colour measurements were determined using a Konica Minolta CM-3600d spectrophotometer (Minolta Co. Ltd., Osaka, Japan) in the CIELab colour space, with the D65 illuminant and 10º observer. The colour coordinates of the beverages were computed in the CIELAB scale in a CIE D65/10° illuminant/observer condition. Colour results were expressed as tristimulus parameters (L^*, a^*, b^*, H^*, C^*). Hue angle

($H^* = \tan^{-1} b^*/a^*$) (2)

Indicates sample colour (0° or 360° = red, 90° = yellow, 180° = green, 270° = blue), and chroma

($C^* = [a^{*2} + b^{*2}]^{1/2}$) (3)

Indicates colour purity or saturation (high values are more vivid); a^* and b^* chromaticity coordinates indicate colour directions green ($-a^*$)/red ($+a^*$) and blue ($-b^*$)/yellow ($+b^*$) (Bakker, Bridle & Timberlake, 1986). Three measurements were taken for each sample.

2.7. Statistical analysis

One-way analysis of variance (ANOVA test) ($p< 0.005$) was used to explore significant differences in anthocyanin composition between sample 0 and the other sample times, using Statistica software (Statsoft, 2004). The IC_{50} was calculated by Graphpad Prism 6 software.

3. Results and discussion

3.1. Identification of anthocyanin compounds by UHPLC- MS/MS Orbitrap

Tables 1 and 2 summarize the retention times (min), molecular formula (M^+), calculated mass and accurate mass, (m/z), accuracy error (ppm), and MS/MS fragments (Accurate mass (m/z), % fragments, calculated mass (m/z), molecular formula, error (ppm)) of a total of 23 compounds identified by UHPLC-MS/MS Orbitrap either matching with available standards or data reported in the literature (tentative identification) when standard were not commercially available.

The anthocyanin profile of strawberry beverages and strawberry purée includes a total of 15 pelargonidin derivates, 4 cyanidin derivates, 3 delphinidin derivates and 1 peonidin derivate (Figure 1). It is the first time that pelargonidin 3-sambubioside, delphinidin 3-arabinoside, cyanidin 3-(6-acetyl)-glucoside and delphinidin 3-galactoside have been tentatively identified in strawberry or a product derived from strawberry. Furthermore, the calculated masses of catechin-(4-8)-pelargonidin 3-glucoside, afzelechin-pelargonidin 3-glucoside, pelargonidin dissacharide (hexose + pentose) acylated with acetic acid, 5-carboxypyranopelargonidin 3-glucoside, pelargonidin 3-arabinoside, pelargonidin 3-malonylglucoside, pelargonidin 3-(6-acetyl)-glucoside and pelargonidin 3-(6-succynil)-arabinoside/ 3-(6-malonyl)-rhamnoside (peaks 1, 2, 11,12,

15, 17, 20 and 23, respectively) are reported as an original contribution of this study (Table 1).

Pelargonidin 3-glucoside (peak 8) was the major compound, followed by pelargonidin 3-rutinoside (peak 9) as showed the positive identification and quantification with standards, in accordance with the literature (Lopes-da-Silva et al., 2002).

Additionally, other pelargonidin derivatives linked to flavonols were identified as follows: catechin-(4-8)-pelargonidin 3-glucoside (peak 1), afzelechin-pelargonidin 3-glucoside and Epi-afzelechin-pelargonidin 3-glucoside (peak 2 and 4 respectively). The latter presented identical accurate masses 705.1796/705.1801 and fragmentation patterns (543/407/313), but different retention times, due to their stereochemistry, which influences overall polarity. Moreover, peaks 11, 14 and 16 correspond to pelargonidin dissacharide (hexose + pentose) acylated with acetic acid. All of them presented the same accurate mass and fragment (*m/z* 271) but different retention times. This might be explained by the presence of different sugar substituents or by a different linkage occurring between the pentose and hexose residues in each of the anomeric carbons of the pigments. For the first time, this contribution reports the tentative identification of pelargonidin 3-sambubioside (peak 7) in a strawberry derivative, with an accurate mass of m/z 565.1552 with -1.3499 ppm of error (Figure 2A). This compound has been identified in other berries, specifically in raspberry extract, with similar high resolution instrumentation (m/z 565.1554) (Mullen, Larcombe, Arnold, Welchman, & Crozier, 2010).

Concerning cyanidin derivates, we identified cyanidin 3-galactoside, cyanidin 3-rutinoside and cyanidin 3-glucoside, all of which have also been detected by Lopes-da-Silva et al. (2007) and Cerezo et al. (2010a) (peaks 5, 6 and 21, respectively).

In addition, the other novelty of this contribution is the tentative identification of cyanidin 3-(6-acetyl)-glucoside (peak 18) (Figure 2B). This compound has been previously described by Brito et al. (2014) in six Chilean berry extracts by HR-ToF-ESI-MS. They found cyanidin 3-O-(6"acetyl)-glucoside in blueberries (*Vaccinium corymbosum*) and berries (*Berberis microphylla*) with an HR-M^+ ion of 491.1206. In addition, this compound has been identified in the skin and wine of the grape Vitis vinifera L. (cv. Aglianico) (De Nisco et al., 2013).

Three derivatives of delphinidin were identified: delphinidin 3-glucoside (peak 22), which was previously reported for the first time by our group in *Camarosa* strawberry purée (Cerezo et al., 2010a); delphinidin 3-arabinoside (m/z 435.0910 and fragment 303) and delphinidin 3-galactoside (peaks 13 and 19, respectively), which have not been described in any strawberry derivative product before (Figures 3 and 4). This anthocyanin compound has been previously identified in other berries (blueberry, blackcurrant and Chilean berry) (Mullen et al., 2010; Sokól-Letowska et al., 2014; Brito et al., 2014).

We could state that all these compounds are naturally present in the strawberry purée and their occurrence in the beverage is not due to the fermentation process (Table 1).

3.2. Effects of temperature on anthocyanin concentration during storage

All the anthocyanin compounds were quantified using the areas of the aglycone counterparts.

Tables 2 and 3 display anthocyanin concentrations of strawberry substrate and gluconic fermented beverages, respectively. Comparing the strawberry substrate sample (before fermentation) with initial sample (0), we observed that the majority of anthocyanin compounds decreased significantly ($p<0.05$). Fermentation process affects severely the anthocyanin composition, decreasing their concentration approximately in a 70%. These results agree with previously published data regarding strawberry wine. Indeed, Klopotek et al. (2005) reported a decrease of 62.4 % after alcoholic fermentation of strawberry mash. An even larger decrease (90.2%) was determined after the fermentation of pomegranate (Orduodi et al., 2014). Conversely, it must be highlighted that certain condensed anthocyanins and also some acetylated ones increased significantly. This is the case for catechin-(4-8)-pelargonidin 3-glucoside, both epi-afzelechin-pelargonidin 3-glucoside and pelargonidin dissacharide (hexose + pentose) acylated with acetic acid. These results are in accordance with Giusti and Wrolstrad (2003) that reported that acylated compounds present a higher stability that monoacylated anthocyanins and they are likely formed during the fermentation process.

Regarding beverages, anthocyanin concentration decreased significantly ($p<0.05$) after 15 days of storage at room temperature. The main compound pelargonidin 3-glucoside decreased significantly, by 37.7%, after 15 days of storage, and by 92.7% at 90 days. Pelargonidin 3-rutinoside and cyanidin 3-galactoside decreased by 89.1 and 99% at 90 days, respectively. A similar trend is shown for the other anthocyanin compounds. Conversely, the most stable compounds were cyanidin 3-glucoside, delphinidin 3-galactoside and 5-carboxypyranopelargonidin 3-glucoside, which decreased by only 34.5, 27.5, and 23.4%, respectively, at room temperature.

On the other hand, when samples were stored at 4 ºC (fridge temperature), the concentrations of the main anthocyanin compounds were preserved until 60 days of storage, while minority compounds such as pelargonidin 3-(6acetyl)-glucoside, pelargonidin 3-(6-succynil)-arabinoside, pelargonidin 3-sambubioside, cyanidin 3- (6-acetyl)-glucoside, cyanidin 3-glucoside and delphinidin 3-arabinoside diminished significantly after 15 days. The percentages of decrease of the major compounds were 34, 17.4 and 54.5% (pelargonidin 3-glucoside, pelargonidin 3-rutinoside and cyanidin 3-galactoside, respectively). The most stable compound was 5-carboxypyranopelargonidin 3-glucoside for both beverages samples (room and fridge temperature). In fact, Andersen, Fossen, Torskangerpoll and Fossen (2004) explained that the pyrano form restricted the formation of the unstable form and the colourless equilibrium in comparison with for ordinary anthocyanins. We can conclude that temperature is crucial in conserving the beverage, with refrigeration being highly recommended to preserve anthocyanin compounds (Tables 2 and 3).

3.1.4. pH, antioxidant activity and colour measurements

Table 4 displays pH and antioxidant activity measured by the ORAC and DPPH and colour parameters of beverages in different storage conditions. The pH does not change under any of these conditions. During storage, in both temperature conditions, a decrease in antioxidant activity was observed for both tests: the higher the storage temperature, the greater the decrease in antioxidant activity. Specifically, in the ORAC test, diminution varies by 10% after 60 days at room temperature. For samples stored at fridge temperature, the diminution is less notable (5%).

The same trend was observed with the DPPH test. In order to achieve 50% DPPH inhibition, higher concentrations of the beverage were required when it was stored at room temperature. Similarly, Wiczkowski, Szawara-Nowak, and Topolska (2015), reported a 6% of decrease in ORAC value of fermented red cabbage after the storage at 4ºC during 90 days that is very close to our results (5.4%). Furthermore, the antioxidant activity of jams made from black carrot (*Daucus carota*) rich in anthocyanins compounds present a more acute decrease when stored at 4º and 20º ranging (8-54%) and (12.8-60.9%) respectively (Kamiloglu, Pasli, Ozcelik, Camp, & Capanoglu, 2015). Therefore, the pH=2.5 that our beverage presents might account for the higher preservation of antioxidant capacity in our work. In fact, a pH=2.5 has been reported to be the best condition for the preservation of strawberry anthocyanins after a 90 days storage conditions (Oliveira et al., 2015).

CIE Lab parameters of the strawberry beverage in different storage conditions were measured. The *L, b* and C values show statistical differences at 15 days, if samples are stored at room temperature. However, when samples are stored at 4 ºC, the significant differences ($p<0.05$) start at 30 days. In addition, for *a* and h values, we observed significant differences from 15 days at both temperatures. Apart from these values, Table 4 shows ΔE ("delta e"), which indicates the difference between two colours in an L*a*b* colour space. This parameter reflects whether the colour differences could be perceptible to the human eye and is calculated as follows:

$$(\Delta E = \sqrt{(\Delta L)^2 + (\Delta a)^2 + (\Delta b)^2}) \quad (4)$$

Where Δ*L*, Δ*a* and Δ*b* are the differences in the *L*, *a* and *b* values between initial and different storage condition samples.

The ΔE classification was based on the one reported by Cserhalmi, Sass-Kiss, Tóth-Markus, and Lechner (2006): 0–0.5 (not noticeable), 0.5–1.5 (slightly noticeable), 1.5–3.0 (noticeable), 3.0–6.0 (well visible) and >6.0 (great). Noticeable differences can be observed after 15 days of storage at room temperature. However, 60 days of storage at fridge temperature are required before appreciable differences can be perceived (4.871).

4. Conclusions

This paper reports for the first time anthocyanins mass with high accuracy (four decimals) of: catechin-(4-8)-pelargonidin 3-glucoside, afzelechin-pelargonidin 3-glucoside, pelargonidin dissacharide (hexose + pentose) acylated with acetic acid, 5-carboxypyranopelargonidin 3-glucoside, pelargonidin 3-arabinoside, pelargonidin 3-malonylglucoside, pelargonidin 3-(6-acetyl)-glucoside and pelargonidin 3-(6-succynil)-arabinoside. Additionally, This contribution reports the tentative identification of four compounds that have not been previously described in strawberry or products made from this fruit: pelargonidin 3-sambubioside, delphinidin 3-arabinoside, cyanidin 3-(6-acetyl)-glucoside and delphinidin3-galactoside based on calculated and accurated mass, error and fragmentation pattern data.,

Storage temperature and time are crucial for conserving the beverage in terms of its anthocyanin profile and colour; consequently, refrigeration is highly recommended to preserve bioactive compounds. If the beverage is stored at 4 ºC, sixty days is the beverage half-life for preserving its composition and properties.

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Figure 1

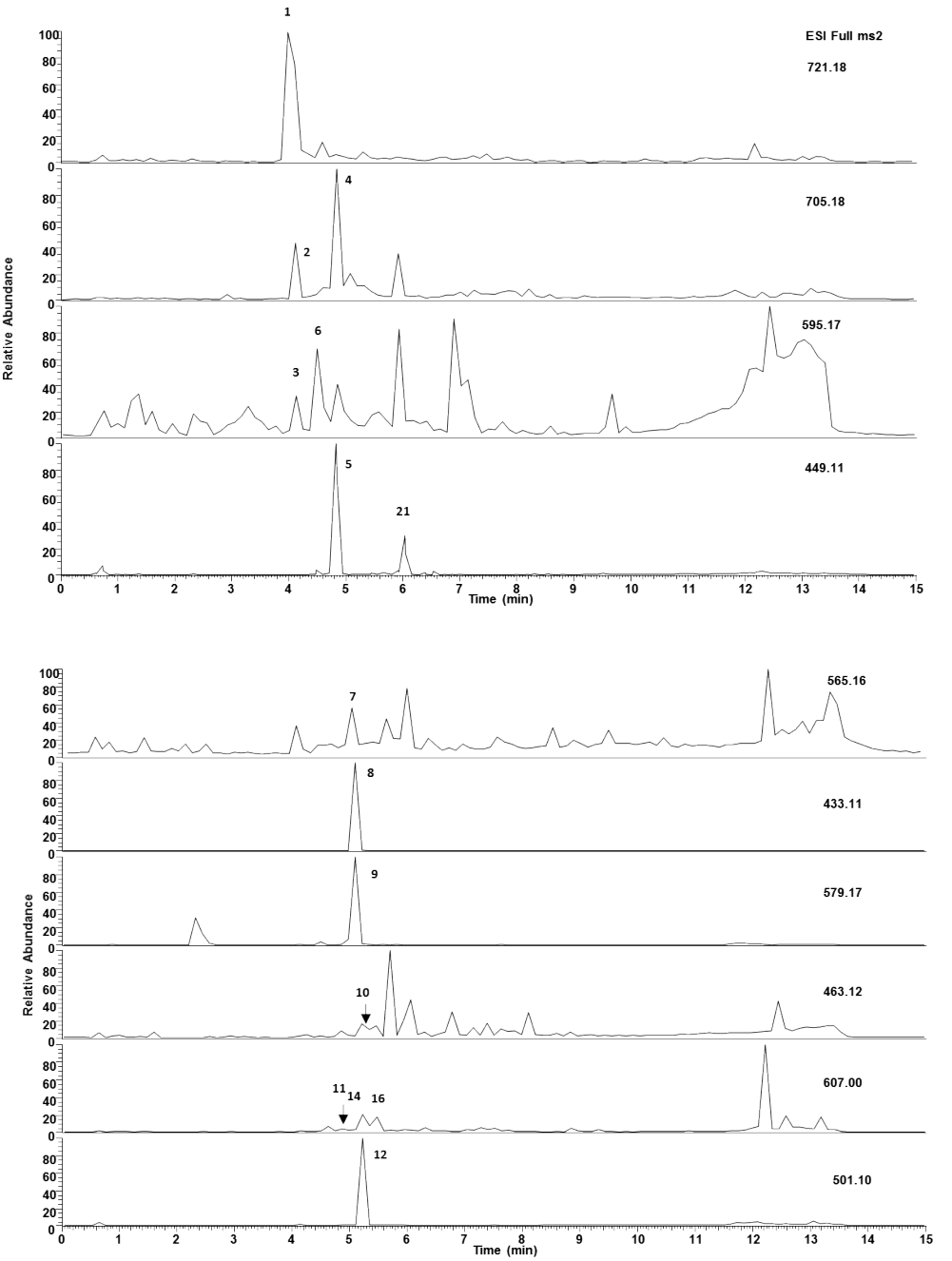

ESI Full ms2
721.18
705.18
595.17
449.11
565.16
433.11
579.17
463.12
607.00
501.10
Relative Abundance
Time (min)
1
2
3
4
5
6
7
8
9
10
11
12
14
16
21

Figure 1: Continuation

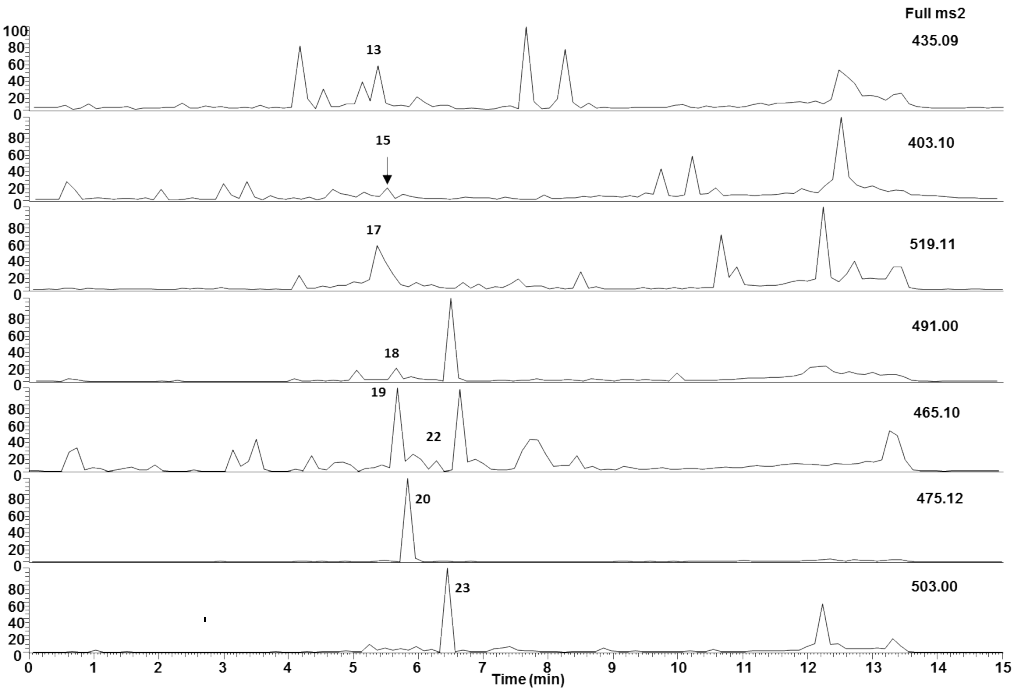


Figure 1: Ion chromatograms from targeted MS2 experiments selecting molecular ions from initial sample. 1: Catechin-(4-8)-pelargonidin 3-glucoside; 2: Afzelechin-pelargonidin 3-glucoside; 3: Pelargonidin 3,5-diglucoside; 4: Epi-Afzelechin-pelargonidin 3-glucoside; 5: Cyanidin 3-galactoside: 6: Cyanidin 3-rutinoside; 7: Pelargonidin 3-sambubioside; 8: Pelargonidin 3-glucoside; 9: Pelargonidin 3-rutinoside; 10: Peonidin 3-glucoside; 11: Pelargonidin dissacharide (hexose + pentose) acylated with acetic acid; 12: 5-carboxypyranopelargonidin 3-glucoside; 13: Delphinidin 3-arabinoside; 14: Pelargonidin dissacharide (hexose + pentose) acylated with acetic acid; 15: Pelargonidin 3-arabinoside; 16: Pelargonidin dissacharide (hexose

+ pentose) acylated with acetic acid; 17: Pelargonidin 3-malonylglucoside; 18: Cyanidin 3-(6-acetyl)-glucoside; 19: Delphinidin 3-galactoside; 20: Pelargonidin 3-(6-acetyl)-glucoside; 21: Cyanidin 3-glucoside; 22: Delphinidin 3-glucoside. 23: Pelargonidin 3-(6-succinyl)-arabinoside/ 3-(6-malonyl)-rhamnoside.

Figure 2

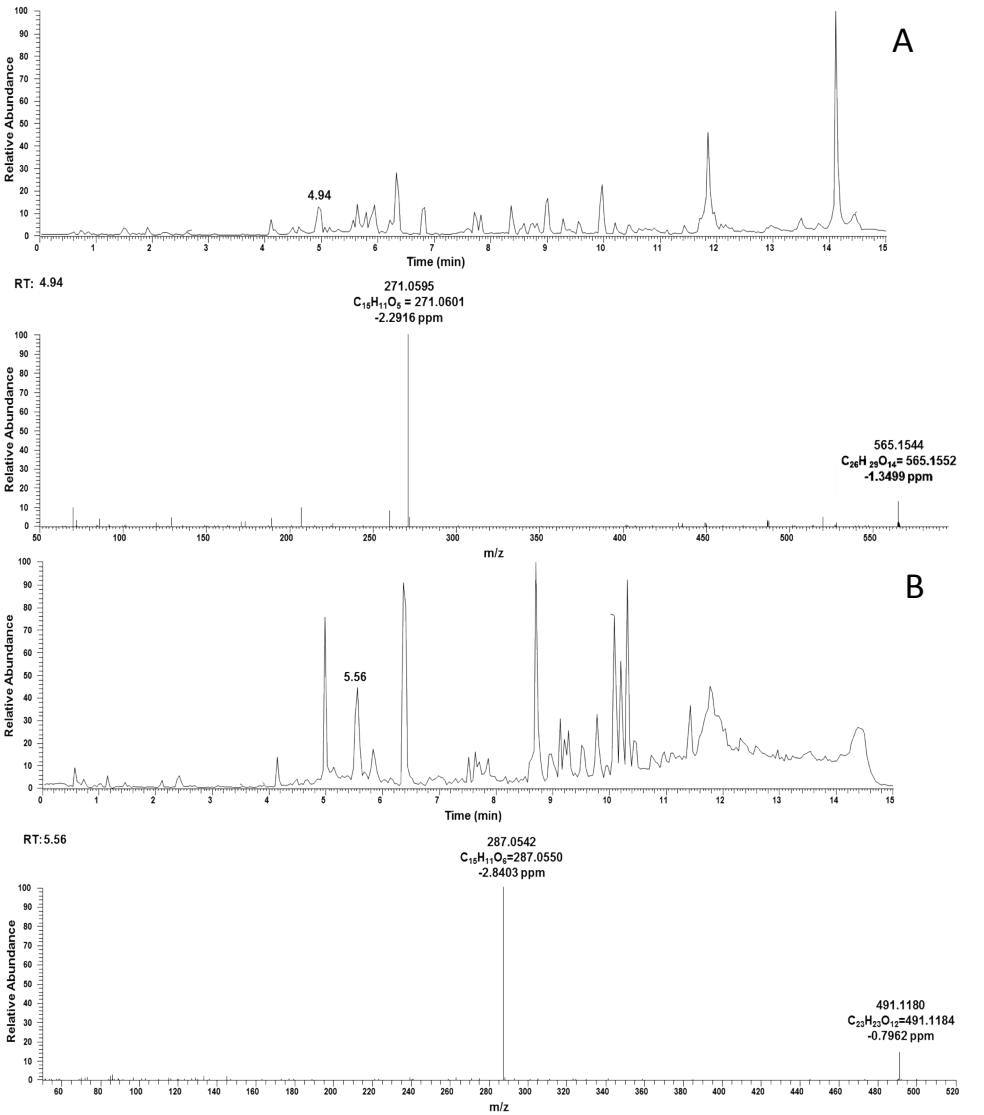


Figure 2: Extracted ion chromatogram (XIC) from targeted-MS2 experiment selecting *m/z* 565.1552 corresponding to pelargonidin 3-sambubioside of initial sample (0) (A

upper) and MS2 spectra (A down). Extracted ion chromatogram (XIC) from targeted-MS2 experiment selecting *m/z* 491.1184 corresponding to cyanidin 3-(6-acetyl)–glucoside of initial sample (0) (B upper) and MS2 spectra (B down).

Figure 3

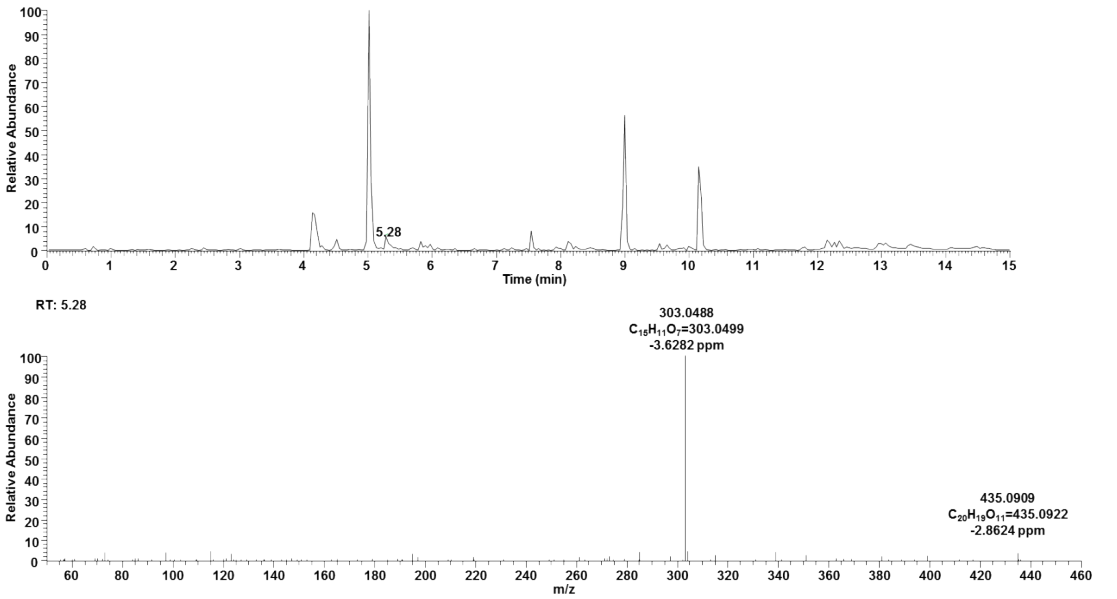


Figure 3: Extracted ion chromatogram (XIC) from targeted-MS2 experiment selecting *m/z* 435.0922 corresponding to delphinidin 3-arabinoside of initial sample (0) (upper) and MS2 spectra (down).

Figure 4

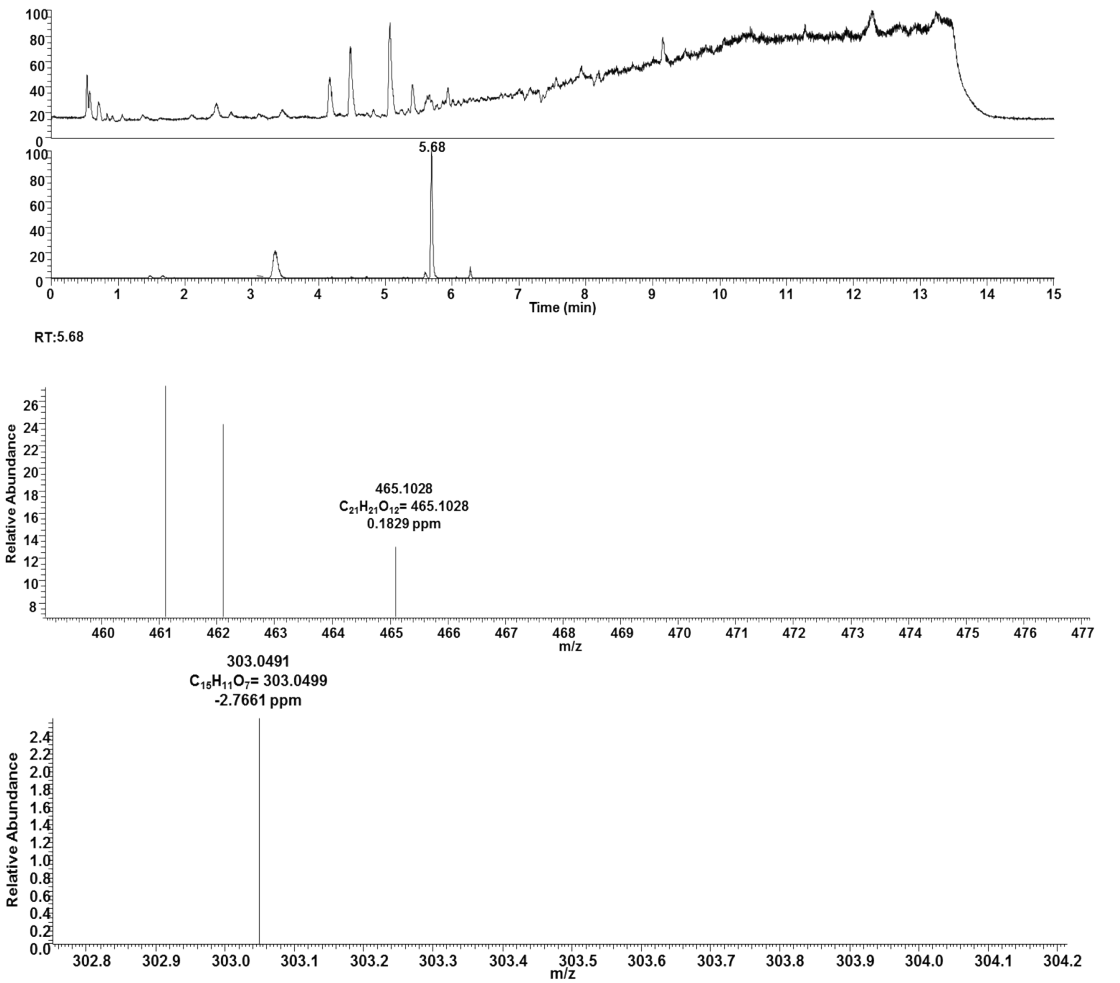


Figure 4: Extracted ion chromatogram (XIC) from full scan experiment selecting *m/z* 465.1028 corresponding to delphinidin 3-galactoside of initial sample (0) (upper) and MS2 spectra (down). Identified by full scan and confirmed with fragmentation pattern.

Table 1: Anthocyanins found in strawberry substrate before fermentation, initial sample and strawberry beverage samples in positive ionization mode. Mean expected retention time (Rt) (min), molecular formula, calculated mass (*m/z*), accurate mass (*m/z*), accuracy error (ppm) and MS/MS fragments (Accurate mass (*m/z*), % fragments, calculated mass (m/z), molecular formula, error (ppm %)).

Peak	Rt (min)	Tentatively identification	Molecular formula (M^+)	Calculated mass (*m/z*)	Accurate mass (*m/z*)	Error (ppm)	MS/MS fragments (Accurate mass (*m/z*), % fragments, calculated mass (*m/z*), molecular formula, error (ppm))	References
1	4.07	Catechin-(4-8)-pelargonidin 3-glucoside	$C_{36}H_{33}O_{16}^+$	721.1763*	721.1744	-2.6814	559.1226 (82.31) / 559.1226/ $C_{30}H_{23}O_{11}^+$/ -1.6621 407.0753 (10.26) / 407.0761/ $C_{22}H_{15}O_8^+$/ -1.9553 313.0698 (7.43) / 313.0707/ $C_{17}H_{13}O_6^+$/ -2.6843	Fossen, Rayyan and Andersen (2004); Cerezo et al. (2010a)
2	4.61	Epi-Afzelechin-pelargonidin 3- glucoside	$C_{36}H_{33}O_{15}^+$	705.1814*	705.1795	-2.6833	543.1277(79.49) / 543.1286/ $C_{30}H_{23}O_{10}^+$/ -1.4365 407.0750 (11.69) / 407.0761/ $C_{22}H_{15}O_8^+$/ -2.7799 313.0698 (8.82) / 313.0707/ $C_{17}H_{13}O_6^+$/ -2.8793	Fossen, Rayyan and Andersen (2004); Cerezo et al. (2010a)
3	4.61	Pelargonidin 3,5-diglucoside	$C_{27}H_{31}O_{15}^+$	595.1657	595.1641	-2.8295	433.1135 (52.82) / 433.1129/ $C_{21}H_{21}O_{10}^+$/ 1.3198 271.0592 (47.18) / 271.0601/ $C_{15}H_{11}O_5^+$/ -3.4175	Lopes-da-Silva et al. (2002) and 2007; Cerezo et al. (2010a)
4	4.76	Epi-Afzelechin-pelargonidin 3- glucoside	$C_{36}H_{33}O_{15}^+$	705.1814*	705.1798	-2.2505	543.1277 (79.53) / 543.1286/ $C_{30}H_{23}O_{10}^+$/ -1.5221 407.0750 (11.72) / 407.0761/ $C_{22}H_{15}O_8^+$/ 2.7050 313.0698 (8.75) / 313.0698/ $C_{17}H_{13}O_6^+$/ -2.8793	Fossen, Rayyan and Andersen (2004); Cerezo et al. (2010a)
5	4.80	Cyanidin 3-galactoside	$C_{21}H_{21}O_{11}^+$	449.1078	449.1074	-0.9941	287.0544 (100) / 287.0550/ $C_{15}H_{11}O_6^+$/ -2.2024	Cerezo et al. (2010a)
6	4.84	Cyanidin 3-rutinoside	$C_{27}H_{31}O_{15}^+$	595.1657	595.1661	0.5547	449.1075 (5.06) / 449.1078/ $C_{21}H_{21}O_{11}^+$/ -0.7544 287.0547 (94.94) / 287.0550/ $C_{15}H_{11}O_6^+$/ -1.1393	Lopes-da-Silva et al. (2002); Wu and Prior (2005); Cerezo et al. (2010a)
7	4.94	Pelargonidin 3-sambubioside	$C_{26}H_{29}O_{14}^+$	565.1552	565.1544	-1.3499	271.0595 (100) / 271.0601/ $C_{15}H_{11}O_5^+$/ -2.2917	a
8	4.96	Pelargonidin 3- glucoside**	$C_{21}H_{21}O_{10}^+$	433.1129	433.1122	-1.7804	271.0596 (100) / 271.0601/ $C_{15}H_{11}O_5^+$/ -1.7287	Lopes-da-Silva et al. (2002); Wu and Prior (2005); Cerezo et al. (2010a)
9	5.08	Pelargonidin 3- rutinoside	$C_{27}H_{31}O_{14}^+$	579.1708	579.1702	-1.1498	433.1118 (4.46) / 433.1129/ $C_{21}H_{21}O_{10}^+$/ -2.5555 271.0594 (95.54) / 271.0601/ $C_{15}H_{11}O_5^+$/ -2.4042	Lopes-da-Silva et al. (2002); Wu and Prior (2005); Cerezo et al. (2010a)
10	5.15	Peonidin 3-glucoside**	$C_{22}H_{23}O_{11}^+$	463.1235	463.1230	-1.0841	301.0699 (100) / 301.0707/ $C_{16}H_{13}O_6^+$/ -2.6899	Cerezo et al. (2010a)
11	5.16	Pelargonidin dissacharide (hexose + pentose) acylated with acetic acid	$C_{28}H_{31}O_{15}^+$	607.1657*	607.1650	-1.2657	271.0597 (100) / 271.0601/ $C_{15}H_{11}O_5^+$/ -1.6161	Lopes-da-Silva et al. (2002); Cerezo et al. (2010a)
12	5.19	5-carboxypyranopelargonidin 3-glucoside	$C_{24}H_{21}O_{12}^+$	501.1028*	501.1018	-1.8262	339.0493 (100) / 339.0499/ $C_{18}H_{11}O_7^+$/ -1.9872	Lopes-da-Silva et al. (2007); Cerezo et al. (2010a)
13	5.28	Delphinidin 3-arabinoside	$C_{20}H_{19}O_{11}^+$	435.0922	435.0909	-2.8624	303.0488 (100) / 303.0499/ $C_{15}H_{11}O_7^+$/ -3.6282	a
14	5.31	Pelargonidin dissacharide (hexose + pentose) acylated with acetic acid	$C_{28}H_{31}O_{15}^+$	607.1657*	607.1657	-0.0594	271.0593 (100) / 271.0601/ $C_{15}H_{11}O_5^+$/ -2.9672	Lopes-da-Silva et al. (2002); Cerezo et al. (2010a)
15	5.40	Pelargonidin 3-arabinoside	$C_{20}H_{19}O_9^+$	403.1024*	403.1719	-1.2935	271.0598 (100) / 271.0601/ $C_{15}H_{11}O_5^+$/ -0.9406	Lopes-da-Silva et al. (2007)
16	5.42	Pelargonidin dissacharide (hexose + pentose) acylated with acetic acid	$C_{28}H_{31}O_{15}^+$	607.1657*	607.1649	-1.4668	271.0592 (100) / 271.0601/ $C_{15}H_{11}O_5^+$/ -3.4175	Lopes-da-Silva et al. (2002); Cerezo et al. (2010a)
17	5.47	Pelargonidin 3-malonylglucoside	$C_{24}H_{23}O_{13}^+$	519.1133*	519.1125	-1.4800	433.1115 (0.39) / 433.1129/ $C_{21}H_{21}O_{10}^+$/ -3.4011 271.0596 (99.61) / 271.0601/ $C_{15}H_{11}O_5^+$/ -1.8413	Lopes-da-Silva et al. (2007)
18	5.56	Cyanidin 3-(6-acetyl)-glucoside	$C_{23}H_{23}O_{12}^+$	491.1184	491.1180	-0.7962	287.0542 (100) / 287.0550/ $C_{15}H_{11}O_6^+$/ -2.8403	a
19	5.68	Delphinidin 3-galactoside	$C_{21}H_{21}O_{12}^+$	465.1028	465.1028	0.1829	303.0488 (100) /303.0499/ $C_{15}H_{11}O_7^+$/ -3.6328	a
20	5.81	Pelargonidin 3-(6-acetyl)-glucoside	$C_{23}H_{23}O_{11}^+$	475.1235*	475.1227	-1.6348	271.0597 (100) / 271.0601/ $C_{15}H_{11}O_5^+$/ -1.3909	Lopes-da-Silva et al. (2007); Cerezo et al. (2010a)
21	5.92	Cyanidin 3-glucoside**	$C_{21}H_{21}O_{11}^+$	449.1078	449.1068	-2.2173	287.0544 (100) / 287.0550/ $C_{15}H_{11}O_6^+$/ -2.3087	Lopes-da-Silva et al. (2002); Wu and Prior (2005); Cerezo et al. (2010a)
22	6.27	Delphinidin 3-glucoside**	$C_{21}H_{21}O_{12}^+$	465.1028*	465.1028	0.1054	303.0491 (100) / 303.0499/ $C_{15}H_{11}O_7^+$/ -2.7661	Cerezo et al. (2010a)
23	6.35	Pelargonidin 3-(6-succinyl)-arabinoside/ 3-(6-malonyl)-rhamnoside	$C_{25}H_{27}O_{11}^+$	503.1548	503.1537	-2.0679	271.0596 (100) / 271.0601/ $C_{15}H_{11}O_5^+$/ -1.3909	Aaby et al. (2007); Cerezo et al. (2010a)

a: non previously reported in strawberry * exact mass reported for the first time in strawberry and strawberry derivate products ** Compounds identified with commercial standards

Table 2: Concentration (µg/L) of anthocyanins present in strawberry substrate before fermentation, initial sample and samples stored at room temperature samples.

Peak	Compound	Strawberry substrate	0	R15	R30	R60	R90
1	Catechin-(4-8)-pelargonidin 3-glucoside	9.31 ± 0.00	13.52 ± 0.08^{a}	9.55 ± 0.00^{b}	7.95 ± 0.06^{c}	2.86 ± 0.01^{d}	1.45 ± 0.01^{e}
2	Epi-Afzelechin-pelargonidin 3-glucoside	7.86 ± 0.00	8.10 ± 0.03^{a}	4.89 ± 0.02^{b}	3.92 ± 0.08^{c}	0.30 ± 0.00^{d}	0.09 ± 0.00^{e}
3	Pelargonidin 3,5-diglucoside	0.40 ± 0.00	0.62 ± 0.00^{a}	0.41 ± 0.01^{b}	0.41 ± 0.06^{c}	0.17 ± 0.01^{d}	0.15 ± 0.02^{e}
4	Epi-Afzelechin-pelargonidin 3-glucoside	2.14 ± 0.08	3.20± 0.08^{a}	1.94 ± 0.07^{b}	1.98 ± 0.04^{c}	0.19 ± 0.01^{d}	0.04 ± 0.00^{e}
5	Cyanidin 3-galactoside	187.95 ± 0.09	61.48 ± 1.22^{a}	31.38 ± 0.77^{b}	21.64 ± 0.08^{c}	4.73 ± 0.10^{d}	0.60 ± 0.10^{e}
6	Cyanidin 3-rutinoside	6.50 ± 0.07	3.61 ± 0.29^{a}	2.50 ± 0.04^{b}	1.96 ± 0.11^{c}	0.79 ± 0.02^{d}	0.48±0.11^{e}
7	Pelargonidin 3-sambubioside	1.11 ± 0.00	1.50 ± 0.11^{a}	1.58 ± 0.03	1.22 ± 0.03^{c}	0.10 ± 0.00^{d}	0.06±0.00^{e}
8	Pelargonidin 3-glucoside	2113.59 ± 29.45	644.77 ± 4.24^{a}	402.41 ± 13.5^{b}	328.54 ± 5.38^{c}	110.85 ± 14.43^{d}	47.26 ± 1.54^{e}
9	Pelargonidin 3-rutinoside	278.04 ± 0.95	62.13 ± 0.11^{a}	50.01 ± 0.69^{b}	42.66 ± 0.13^{c}	13.48 ± 0.05^{d}	6.79 ± 0.24^{e}
10	Peonidin 3-glucoside	5.65 ± 0.04	1.10 ± 0.02^{a}	0.66 ± 0.02^{b}	0.53 ± 0.01^{c}	0.20 ± 0.00^{d}	0.19 ± 0.00^{e}
11	Pelargonidin dissacharide (hexose + pentose) acylated with acetic acid	1.35 ± 0.01	1.40 ± 0.08^{a}	0.78 ± 0.02^{b}	0.11 ± 0.00^{c}	0.07 ± 0.00^{d}	0.06 ± 0.00^{e}
12	5-carboxypyranopelargonidin 3-glucoside	66.31 ± 0.53	16.03 ± 0.65^{a}	14.38 ± 1.24	18.15 ± 0.44^{c}	17.56 ± 1.11	12.27 ± 0.43^{e}
13	Delphinidin 3-arabinoside	2.60 ± 0.15	5.24 ± 0.08^{a}	3.57 ± 0.01^{b}	3.27 ± 0.04^{c}	2.20 ± 0.01^{d}	1.75 ± 0.21^{e}
14	Pelargonidin dissacharide (hexose + pentose) acylated with acetic acid	0.88 ± 0.00	1.39 ± 0.02^{a}	0.79 ± 0.00^{b}	0.05 ± 0.00^{c}	0.03 ± 0.00^{d}	0.05 ± 0.00^{e}
15	Pelargonidin 3-arabinoside	0.84 ± 0.00	0.62 ± 0.06^{a}	0.30 ± 0.04^{b}	0.24 ± 0.01^{c}	0.03 ± 0.00^{d}	0.09 ± 0.01^{e}
16	Pelargonidin dissacharide (hexose + pentose) acylated with acetic acid	0.92 ± 0.00	0.61 ± 0.02^{a}	0.34 ± 0.01^{b}	0.35 ± 0.00^{c}	0.20 ± 0.01^{d}	0.13 ± 0.00^{e}
17	Pelargonidin 3-malonylglucoside	124.90 ± 0.58	21.45 ± 0.45^{a}	12.21 ± 0.71^{b}	8.16 ± 0.73^{c}	1.60 ± 0.08^{d}	1.39 ± 0.02^{e}
18	Cyanidin 3-(6-acetyl)-glucoside	1.83 ± 0.00	3.88 ± 0.05^{a}	2.12 ± 0.02^{b}	1.66 ± 0.22^{c}	1.00 ± 0.02^{d}	0.91 ± 0.00^{e}
19	Delphinidin 3-galactoside	36.98 ± 0.01	20.94 ± 1.54^{a}	15.20 ± 0.12^{b}	14.80 ± 0.02^{c}	14.60 ± 0.05^{d}	15.17 ± 0.06^{e}
20	Pelargonidin 3-(6-acetyl)-glucoside	149.75 ± 0.02	36.34 ± 0.13^{a}	18.48 ± 0.16^{b}	14.59 ± 0.12^{c}	3.00 ± 0.02^{d}	0.81 ± 0.02^{e}
21	Cyanidin 3-glucoside	86.59 ± 0.01	41.02 ± 0.28^{a}	29.19 ± 0.09^{b}	27.05 ± 0.20^{c}	27.79 ± 0.11^{d}	26.85 ± 0.92^{e}
22	Delphinidin 3-glucoside	1.05 ± 0.00	1.69 ± 0.07^{a}	1.51 ± 0.03^{b}	1.23 ± 0.02^{c}	0.76 ± 0.04^{d}	0.81 ± 0.01^{e}
23	Pelargonidin 3-(6-succinyl)-arabinoside/ 3-(6-malonyl)-rhamnoside	32.86 ± 0.06	10.19 ± 0.10^{a}	5.27 ± 0.02^{b}	3.97 ± 0.17^{c}	0.95 ± 0.07^{d}	0.49 ± 0.00^{e}

^{a,b,c,d,e}Different subscripts letters means significant differences ($p<0.05$) between the samples as follows a (strawberry purée and 0); b (0 and 15); c (15 and 30); d (30 and 60) e (60 and90).

Table 3: Concentration (µg/L) of anthocyanins present in strawberry substrate before fermentation, initial sample and samples stored at fridge temperature samples

Peak	Compound	Strawberry substrate	0	F15	F30	F60	F90
1	Catechin-(4-8)-pelargonidin 3-glucoside	9.31 ± 0.00	13.52 ± 0.08^{a}	12.20 ± 0.22^{b}	12.57 ± 0.02	7.57 ± 0.07^{d}	6.62 ± 0.12^{e}
2	Epi-Afzelechin-pelargonidin 3-glucoside	7.86 ± 0.00	8.10 ± 0.03^{a}	7.48 ± 0.22^{b}	7.95 ± 0.37	3.55 ± 0.23^{d}	3.41 ± 0.01^{e}
3	Pelargonidin 3,5-diglucoside	0.40 ± 0.00	0.62 ± 0.00^{a}	0.64 ± 0.02	0.64 ± 0.02	0.47 ± 0.02^{d}	0.48 ± 0.02^{e}
4	Epi-Afzelechin-pelargonidin 3-glucoside	2.14 ± 0.08	3.20± 0.08^{a}	3.12 ± 0.12	3.19 ± 0.10	1.91 ± 0.05^{d}	1.57 ± 0.01^{e}
5	Cyanidin 3-galactoside	187.95 ± 0.09	61.48 ± 1.22^{a}	56.83 ± 1.96^{b}	45.23 ± 0.16^{c}	29.74 ± 0.14^{d}	22.62 ± 0.10^{e}
6	Cyanidin 3-rutinoside	6.50 ± 0.07	3.61 ± 0.29^{a}	3.10 ± 0.04^{b}	3.17 ± 0.05	1.87 ± 0.08^{d}	1.65 ±0.15^{e}
7	Pelargonidin 3-sambubioside	1.11 ± 0.00	1.50 ± 0.11^{a}	1.03 ± 0.00^{b}	0.95 ± 0.04^{c}	0.15 ± 0.00^{d}	0.15 ±0.00^{e}
8	Pelargonidin 3-glucoside	2113.59 ± 29.45	644.77 ± 4.24^{a}	603.27 ± 7.74^{b}	621.36 ± 2.97^{c}	425.27 ± 16.61^{d}	405.33 ± 1.25^{e}
9	Pelargonidin 3-rutinoside	278.04 ± 0.95	62.13 ± 0.11^{a}	72.55 ± 3.50^{b}	70.14 ± 2.05^{c}	51.35 ± 0.59^{d}	46.60 ± 0.58^{e}
10	Peonidin 3-glucoside	5.65 ± 0.04	1.10 ± 0.02^{a}	1.07 ± 0.00	1.00 ± 0.02^{c}	0.66 ± 0.02^{d}	0.55 ± 0.03^{e}
11	Pelargonidin dissacharide (hexose + pentose) acylated with acetic acid	1.35 ± 0.01	1.40 ± 0.08^{a}	1.38 ± 0.07	1.40 ± 0.02	0.97 ± 0.05^{d}	0.88 ± 0.07^{e}
12	5-carboxypyranopelargonidin 3-glucoside	66.31 ± 0.53	16.03 ± 0.65^{a}	19.69 ± 1.19^{b}	19.12 ± 1.14	18.94 ± 0.67^{d}	18.62 ± 0.99
13	Delphinidin 3-arabinoside	2.60 ± 0.15	5.24 ± 0.08^{a}	4.31 ± 0.06^{b}	4.03 ± 0.00^{c}	3.99 ± 0.25^{d}	3.85 ± 0.07^{e}
14	Pelargonidin dissacharide (hexose + pentose) acylated with acetic acid	0.88 ± 0.00	1.39 ± 0.02^{a}	1.12 ± 0.03^{b}	0.95 ± 0.02^{c}	0.76 ± 0.10^{d}	0.68 ± 0.01^{e}
15	Pelargonidin 3-arabinoside	0.84 ± 0.00	0.62 ± 0.06^{a}	0.50 ± 0.00^{b}	0.52 ± 0.01	0.24 ± 0.00^{d}	0.09 ± 0.00^{e}
16	Pelargonidin dissacharide (hexose + pentose) acylated with acetic acid	0.92 ± 0.00	0.61 ± 0.02^{a}	0.59 ± 0.01	0.35 ± 0.00^{c}	0.38 ± 0.00^{d}	0.38 ± 0.01^{e}
17	Pelargonidin 3-malonylglucoside	124.90 ± 0.58	21.45 ± 0.45^{a}	21.45 ± 0.28	24.24 ± 0.19^{c}	14.05 ± 0.84^{d}	10.22 ± 0.94^{e}
18	Cyanidin 3-(6-acetyl)-glucoside	1.83 ± 0.00	3.88 ± 0.05^{a}	3.08 ± 0.19^{b}	2.67 ± 0.07^{c}	1.62 ± 0.01^{d}	1.29 ± 0.13^{e}
19	Delphinidin 3-galactoside	36.98 ± 0.01	20.94 ± 1.54^{a}	17.86 ± 0.00^{b}	17.56 ± 0.34^{c}	16.07 ± 0.64^{d}	16.05 ± 0.89^{e}
20	Pelargonidin 3-(6-acetyl)-glucoside	149.75 ± 0.02	36.34 ± 0.13^{a}	29.48 ± 0.01^{b}	35.52 ± 0.08^{c}	17.16 ± 0.30^{d}	17.36 ± 0.46^{e}
21	Cyanidin 3-glucoside	86.59 ± 0.01	41.02 ± 0.28^{a}	35.66 ± 0.13^{b}	34.85 ± 0.21^{c}	35.51 ± 0.08^{d}	31.74 ± 0.81^{e}
22	Delphinidin 3-glucoside	1.05 ± 0.00	1.69 ± 0.07^{a}	1.39 ± 0.00^{b}	1.05 ± 0.01^{c}	1.08 ± 0.08^{d}	1.07 ± 0.09^{e}
23	Pelargonidin 3-(6-succinyl)-arabinoside/ 3-(6-malonyl)-rhamnoside	32.86 ± 0.06	10.19 ± 0.10^{a}	9.24 ± 0.03^{b}	8.97 ± 0.17^{c}	5.39 ± 0.11^{d}	4.71 ± 0.02^{e}

^{a,b,c,d,e} Different subscripts letters means significant differences ($p<0.05$) between the samples as follows a (strawberry purée and 0); b (0 and 15); c (15 and 30); d (30 and 60) e (60 and90).

Table 4: pH, antioxidant activity and color measurements of strawberry fermented beverage.

Sample	pH	ORAC (mmol Trolox/ mL beverage)	DPPH (IC50)	L*	a*	b*	C*	h	ΔE
0	2.52	107.810 ± 1.010	1.993 (1.783-2.228)*	77.814 ± 0.001	21.184 ± 0.055	27.148 ± 0.005	34.435 ± 0.037	52.035 ± 0.067	
R15	2.53	86.561 ± 3.169 [a]	2.463 (2.154-2.817)	79.095 ± 0.001[a]	17.656 ±0.009[a]	25.914 ± 0.005[a]	31.357 ± 0.001[a]	55.733 ± 0.019[a]	3.951
R30	2.53	101.004 ± 1.395[a]	2.531 (2.330-2.750)	78.957 ± 0.005[a]	14.744 ±0.031[a]	26.603 ± 0.004[a]	30.415 ± 0.018[a]	61.005 ± 0.047[a]	6.563
R60	2.53	97.257 ± 2.039[a]	2.513 (2.350-2.687)	78.795 ± 0.067[a]	10.843 ±0.035[a]	28.703 ± 0.129[a]	30.682 ± 0.133[a]	69.304 ± 0.234[a]	10.502
R90	2.53	96.596 ± 1.517[a]	3.638 (3.396-3.897)	79.668 ± 0.031[a]	9.068 ± 0.022[a]	29.015 ± 0.001[a]	30.399 ± 0.005[a]	72.645 ± 0.040[a]	12.398
F15	2.52	137.174 ± 1.980[a]	0.7443 (0.5190-1.067)	77.828 ± 0.025	20.895 ±0.064[a]	27.159 ± 0.016	34.267 ± 0.052	52.427 ± 0.069[a]	0.289
F30	2.53	123.105 ± 0.163[a]	0.7951 (0.5004-163.2)	78.674 ± 0.002[a]	20.943 ±0.010[a]	26.227 ± 0.020[a]	33.563 ± 0.022[a]	51.391 ± 0.008[a]	1.283
F60	2.52	102.763 ± 1.617[a]	1.1700 (1.007-1.359)	79.584 ± 0.006[a]	17.098 ±0.056[a]	25.172 ± 0.045[a]	30.430 ± 0.068[a]	55.814 ± 0.039[a]	4.871
F90	2.54	101.904 ± 2.331[a]	1.467 (1.326-1.622)	79.049 ± 0.046[a]	16.616 ±0.002[a]	25.860 ± 0.026[a]	30.738 ± 0.023[a]	57.278 ± 0.023[a]	4.904

[a] Subscripts mean significant differences ($p<0.05$) with respect to initial samples.

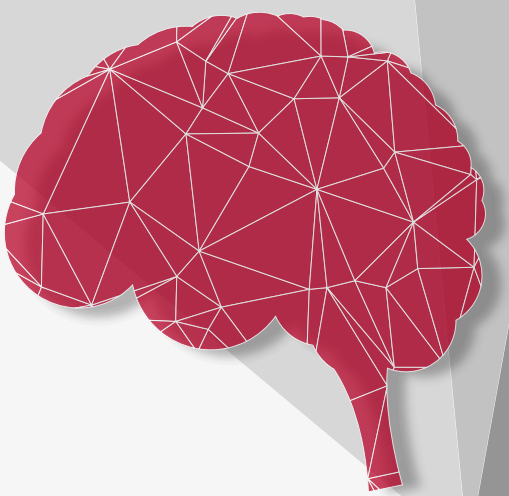
*Confidence Interval expressed in brackets

L^*: lightness. a^* and b^* chromaticity coordinates indicate color directions green ($-a^*$)/red ($+a^*$) and blue ($-b^*$)/yellow ($+b^*$). $C^* = [a^{*2} + b^{*2}]^{1/2}$ indicates color purity or saturation. $H^* = \tan^{-1} b^*/a^*$ indicates sample color.
ΔE: difference between two colors in an L*a*b* color space.

Capítulo 4

Ácido protocatéquico: inhibición de la formación y desestabilización de fibras de β-amiloide y α-sinucleína y propiedades neuroprotectoras.

Protocatechuic acid: inhibition of the fibril formation, destabilization of pre-formed fibrils of amyloid-β and α-synuclein and neuroprotection.



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Protocatechuic acid: inhibition of fibril formation, destabilization of pre-formed fibrils of Amyloid-β and α-Synuclein, and neuroprotection

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Abstract

Protocatechuic acid (PCA) is the major metabolite of the anthocyanin known as cyanidin 3-glucoside. It is found in plasma and tissues, such as the brain, heart, liver and kidneys, following consumption of a rich source of this flavonoid. The abnormal pathological assembly of Amyloid-β (Aβ) and α-Synuclein (αS) is an underlying mechanism involved in the formation of amyloid plaques and Lewy bodies in the brain, which are responsible for neuropathology symptoms in Alzheimer's (AD) and Parkinson's Diseases (PD), respectively. This research was performed to evaluate the protective effects of PCA, by establishing its potential role in inhibiting aggregation and fibril destabilization of Aβ and αS proteins. It has been found that PCA inhibits the aggregation of Aβ and αS, and destabilizes their pre-formed fibrils. These results were confirmed by TEM images, electrophoresis and immunoblotting experiments. Furthermore, PCA prevents the death of PC12 cells triggered by Aβ- and αS-induced toxicity.

Keywords: neurodegeneration, toxicity, protocatechuic acid, Amyloid-β, α-Synuclein.

Introduction

Berries and red wine are an excellent source of anthocyanin compounds, which have attracted interest because they are responsible for their color.[1] Furthermore, certain healthy effects associated with consuming these products have been attributed to these compounds.[2]

However, different studies have shown that 69% of the anthocyanins present in foods disappear from the gastrointestinal tract within 4 hours following food ingestion.[3] Furthermore, several *in vitro* and animal studies suggest that some of them disappear due to the degradation of anthocyanins aglycones into the corresponding phenolic acids.[4–7] Indeed, anthocyanins present a low recovery rate after ingestion, less than 1%.[8] Recently, an isotope-labeled cyanidin 3-glucoside human feeding study showed higher recoveries, of about 12.4%, if recovery of the metabolites formed is also considered.[9] Protocatechuic acid (PCA) is the major phenolic acid metabolite formed from anthocyanins.[10, 11] Additionally, PCA is present in foods in varying quantities: berries (0.62–794.13 mg/100 g), red wine (0.21 mg/100 mL), fruits (0.28–18.73 mg/100 g), jams and berry jams (0.07–9.36 mg/100 g), fruit juices (1.14–6.70 mg/100 mL, vegetables (0.62–10.62 mg/100 g).[12]

Therefore, PCA is of particular nutritional interest because it can reach tissues in quantities that can have biological effects on health.[13] *In vivo* studies have demonstrated that following ingestion of a standard diet supplemented with PCA (2–4 g) for 12 weeks, there was an increase of PCA levels in plasma and tissues, such as the brain, heart, liver and kidneys.[14] Moreover, PCA has been identified in rat plasma and tissues, and in human blood, following administration of cyanidin 3-glucoside.[10]

Furthermore, PCA has been detected in blood a few minutes after ingestion, a fact that can explain the acute increase of plasma antioxidant activity found in humans following ingestion of foods containing anthocyanins.[15] Therefore, potential effects of such metabolites should be taken into account when assessing the health benefits of anthocyanins.

Recently, several investigations have shown that PCA presents a variety of health benefits, partly attributed to its catechol structure, due to its anti-inflammatory properties,[16,17] antioxidant and free radical scavenging activities,[18–19] antidiabetic effects[20, 21, 14] and neuroprotective properties.[22–25]

Alzheimer's (AD) and Parkinson's Disease (PD) are the most common human neurodegenerative disorders. Protein aggregation, which results in different fibrillar structures, is responsible for neuropathology symptoms, due to the abnormal assembly of Amyloid-β (Aβ) and α-Synuclein (αS). The pathological aggregation process of both proteins consists of consecutive steps, which begin when the soluble forms of the proteins (which display the capacity to assemble themselves) lead to the establishment of toxic protofibril aggregates, which are responsible for the neurotoxic effects. Indeed, several studies have demonstrated that protofibrils and oligomers of Aβ and αS are more neurotoxic than fibrils.[26-29]

In this study, the effects of PCA on the aggregation and destabilization processes of Aβ and αS were investigated. Furthermore, its cytoprotective effect against Aβ- and αS-induced toxicity was monitored. For this purpose, we have used different techniques: ThT assay, electrophoresis and Western blot analysis, transmission electron microscopy (TEM), and cell viability (MTT).

Materials and methods

Chemicals

Standards and reagents were purchased from the following suppliers: Sigma Aldrich, Steinheim, Germany (Protocatechuic acid, Thioflavin T [ThT], Dimethyl sulfoxide [DMSO], Dulbecco's Modified Eagle's Medium [DMEM]-Glutamax, Trypsine-EDTA, Thiazolyl Blue Tetrazolium Bromide [MTT], Phosphate Buffered Saline [PBS], L-glutamine, fetal horse serum, fetal bovine serum, and streptomycin); Alexotech, Umeå, Sweden ($A\beta_{1-42}$ and αS); Panreac, Castellar del Vallès, Barcelona, Spain (Na_2HPO_4/NaH_2PO_4 and NaCl); ATCC, Manassas, USA (PC12 cells); IBL, Minneapolis, Minnesota (Anti-human amyloid-B [N] [82E1] mouse IgG MoAb); Covance, Japan (Beta Amyloid, 1–16 [6E10] monoclonal antibody); Cell Signaling Technology, Danvers, Massachusetts (Anti-mouse IgG, HRP-linked antibody); Bioline, London, UK (HyperPAGE Prestained Protein Marker); Applichem, Darmstadt, Germany (SDS-Solution 10% [v/v], glycine, bromophenol blue and glycerol anhydrous); Bio-Rad, Munich, Germany (2-Mercaptoetanol, 10x Tris/Glycine/SDS [10x premixed electrophoresis buffer contains 25 mM], Tris 192 mM Glycine [0.1% SDS, pH 8.3], 10x Tris/Glycine [10x premixed electrophoresis buffer, pH 8.3], 4–20% polyacrylamide Stain-Free Gel Mini-PROTEAN® TGX, Immun-Blot PVDF membrane, and Coomassie Blue); Thermo Scientific, Rockford, USA (Pierce, ECL 2 Plus Western Blotting Substrate); EMS, Hatfield, Pennsylvania, United States (Carbon-coated grids [300 mesh, copper]).

Measurement of Aβ and αS fibril formation and destabilization assay (ThT assay)

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A stock solution of 221 µM of $A\beta_{1-42}$ protein was prepared in buffer Na_2HPO_4/NaH_2PO_4 (25 mM)/NaCl (140 mM), adjusted to pH 7.4, and then diluted to 20 µM.A stock solution of PCA was prepared at 50 mM in DMSO, and subsequently diluted with buffer to 1000 µM. From this solution, 4 PCA solutions were prepared, with the following concentrations: 20, 40, 100 and 200 µM.The aggregation and destabilization experiments were performed according to Ono et al.[30, 31] with slight modifications: in this experiment we used fluorescent molecule ThT. A solution stock was prepared at a concentration of 1 mM, in phosphate buffer; it was then filtered and refrigerated. Next, equal volumes of each solution were mixed with 20 µM of $A\beta_{1-42}$ and put on black, clear-bottom, 96-well plates. 3 µL of ThT were added to each well (25 µM per well, at final concentration). Fluorescence emission data were recorded every 2 hours, for a 48-hour period, using a multi-detector microplate reader (Synergy HT, Biotek®) fluorescence spectrophotometer, set at 450 nm for excitation and 485 nm for emission wavelengths. Assay of the disaggregation of pre-formed fibrils of Aβ requires fibrils to have been formed; consequently, 20 µM of Aβ solution were incubated for 3 days, at 37°C, under continuous agitation. Similarly, equal volumes of Aβ fibrils were mixed with PCA, at different concentrations. Measurements were recorded as explained above. The aggregation and destabilization experiments were conducted for αS in the same way as described for Aβ, except the concentration of αS was 140 µM in the stock solution, diluted to 70 µM, and the experiments lasted for 6 days, at 37°C, without agitation for destabilization. At least 3 experiments were performed for each test (4), at 4 concentrations, in triplicate (3), resulting in 144 samples, which were frozen at -80°C and subsequently used for further experiments: electrophoresis, Western blot analysis and Transmission Electron Microscopy (TEM).

PC12 cell culture

PC12 cells (rat pheochromocytoma cells) were obtained from the American Type Culture Collection (ATCC, Manassas, USA). They were cultured in 75 cm^2 culture flasks, containing 20 mL of DMEM-Glutamax, supplemented with 100 IU/mL of penicillin, 100 µg/mL, streptomycin, 15% (v/v) of fetal horse serum and 2.5% (v/v) of fetal bovine serum, at 37°C, with 5% CO_2. Cells were detached every 3 days using 1X Trypsin-EDTA. 96-well plates were used for the cell viability experiments, at a density of 30,000 cells per well.

Cell cytotoxicity (MTT assay)

Dilutions of PCA (2, 5, 10, 20, 50 and 100 µM) were prepared from stock solutions, with serum-free culture medium. Equal volumes of each solution were mixed with $A\beta_{1-42}$ (10 µM), then incubated for 24 hours on a thermoblock, with continuous agitation, and then exposed to PC12 cells for 24 hours, to test whether PCA can prevent cell death triggered by Aβ. Cell viability was determined by MTT reduction assay.[32] Cells were treated with 200 µL per well of MTT solution (final concentration, 0.5 mg/mL in DMEM-Glutamax medium) for 3 hours, at 37°C, with 5% CO_2. The dark blue formazan crystals that formed were solubilized with 100 µL per well of DMSO, for 30 minutes. Absorbance was measured at 540 nm, with a microplate reader (Synergy HT, Biotek®). Results were expressed as the percentage of MTT reduction in relation to the absorbance of control cells at 100%.

In the same way, αS and PCA were diluted with serum-free culture medium, to achieve 7 µM (αS) and 1, 5, 25 and 50 µM (PCA) at final concentrations, and incubated for 6 days, on a thermoblock, with continuous agitation (pretreatment). Samples taken at 2

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157 days and at 6 days were exposed to PC12 cells for 48 hours, to test the protective
158 effect of this compound in relation to cell death, in the protein's different states.
159 Finally, MTT treatment was performed, as explained above.

160 **Electrophoresis and Western blot analysis**

161 To confirm the effect of PCA on inhibition of the fibril formation of Aβ protein,
162 electrophoresis and Western blot analysis were performed. 15 µL of ThT samples were
163 diluted with 5 µL of loading buffer, for inhibition of the aggregation assay. For the
164 destabilization of fibrils assay, 10 µL of sample were diluted with 10 µL of loading
165 buffer, which had the following composition: Tris-Base (0.125M), Glycerol (20% v/v),
166 SDS (4%), 2-β-mercaptoethanol (10% v/v) and bromophenol blue (0.06% w/v) (Bio-
167 Rad). After gel electrophoresis (4–20% precast polyacrylamide, Bio-Rad) the proteins
168 were transferred to polyvinylidene fluoride (PVDF) membranes, by applying 310 mA
169 for at least 1 hour, on a Mini-PROTEAN Tetra Cell, from Bio-Rad. The membrane was
170 boiled for 5 minutes with PBS (1x) and then rapidly cooled. The membrane was then
171 incubated with a mix of primary monoclonal antibodies 6E10 and 82E1 (at 1/6000
172 dilution), overnight at 4°C. Afterwards, the samples were cleaned with TBST, 3 times,
173 for 5 minutes, and incubated with anti-mouse IgG antibody (1/10000), for 1 hour.
174 Following this, the membrane was cleaned with TBST, 3 times, for 5 minutes, and
175 examined using chemiluminescence (Amersham Imager 600, GE Healthcare Life
176 Sciences).

177 To confirm the effects of PCA on inhibition of αS fibril formation and destabilization of
178 pre-formed fibrils, 15 µL of ThT samples were diluted with 5 µL of loading buffer, as
179 described earlier. Samples were boiled at 50°C for 3 minutes, and loaded on 4–20%

Tris-Glycine gel, for 1 hour, at 100 volts. Next, the gels were stained with Coomassie Blue (0.1% Coomassie R250, 10% acetic acid, and 40% methanol).

TEM images

10 µL of samples obtained in the experiments explained in section 2.2 ($A\beta_{1-42}$ [10 µM] or αS [70 µM], with PCA at 10, 20, 50 and 100 µM) were placed on a 300 mesh carbon-coated Formvard grid, and incubated for 20 minutes. Subsequently, excess fluid was removed, and 5 µL of 2.5% glutaraldehyde (v/v) were placed on the grid, and it was incubated for an additional 5 minutes. Later, the grids were negatively stained for 1 minute, with 5 µL of 0.5% uranyl acetate solution. Excess fluid was removed, and the samples were viewed using a Zeiss Libra 120 TEM, operating at 80 Kv.

Statistical analysis

One-way analysis of variance (ANOVA test) ($p<0.05$) was used for cell viability measurements.[33]

Results and discussion

Effects of PCA on $A\beta_{1-42}$ fibril formation

Inhibition of $A\beta_{1-42}$ aggregation by PCA was measured by ThT fluorescence assay. Figure 1A displays the ΔThT fluorescence induced by $A\beta_{1-42}$ aggregation, when incubated alone, showing that 30 hours is the time required to obtain amyloid fibrils. Figure 1B represents ΔThT fluorescence when $A\beta_{1-42}$ was incubated with 4 different concentrations of PCA (10, 20, 50 and 100 µM), for 48 hours, the percentages of inhibition being 30, 40, 63 and 79%, respectively. Whatever the concentration, a delay

was observed between the addition of PCA and effective observation of inhibition of aggregation. It is interesting to highlight that the time required to achieve an inhibition effect is influenced by the concentration of PCA: the lower the concentration, the longer the time required. 20 hours were necessary to observe a decrease of fluorescence at 10 and 20 µM. Conversely, for 50 and 100 µM, the decrease of fluorescence began after 10 hours of experiment.

TEM was used to observe the aggregation state of $A\beta_{1-42}$ at different concentrations of PCA, after 48 hours of incubation. When $A\beta_{1-42}$ was incubated alone for 48 hours, numerous aggregates and fibrils were observed (Figure 1C); whereas, for the samples incubated with PCA, the aggregate number decreased as PCA concentration increased (Figures 1D to 1G). Conversely, incubation with 50 and 100 µM of PCA showed that the aggregates practically disappeared (Figures 1F and 1G). Consequently, a dose-dependent effect has been confirmed.

Electrophoresis was used to investigate the species formed in the presence of PCA. Western blot analysis, using a specific antibody that recognizes small Aβ oligomers, revealed the emergence of protein species with different molecular weights (Figure 1H). In particular, after incubation with 100 µM of PCA, the bands corresponding to monomeric (3.5 kDa), dimeric and trimeric (10–15 kDa) species were more intense. Moreover, bands corresponding to dimers and trimers (10–15 kDa) were observed. In summary, the whole set of experiments performed demonstrates the inhibitory effect of PCA on $A\beta_{1-42}$ aggregation, in a dose-dependent manner.

Effects of PCA on destabilization of pre-formed fibrils of Aβ

ThT assays with pre-formed fibrils of Aβ (fAβ) were performed to test whether PCA can destabilize pre-formed fibrils. To this end, fAβ were incubated with four different concentrations of PCA (10, 20, 50 and 100 µM).

Figure 2A shows that PCA interacted with the fibrils from the beginning of the experiment, and destabilized fAβ in a dose-dependent manner, the percentages of destabilization being 35, 53, 55 and 65%, for 10, 20, 50 and 100 µM, respectively.

To confirm the destabilization of pre-formed fibrils, TEM experiments were performed. Accordingly, Figure 2B shows the fibrils formed when Aβ was incubated alone. Noticeable differences are observed in Figure 2C (incubation with 10 µM of PCA), while incubation with 20, 50 and 100 µM of PCA led to smaller aggregates (Figures 2D, 2E and 2F).

Similarly, electrophoresis and immunoblotting analysis (Fig. 2G) revealed that, at higher concentrations, the bands corresponding to small monomers with a low molecular weight present more intensity, which proves that PCA destabilizes fibrils that have already been formed.

Effects of PCA against $A\beta_{1-42}$ toxicity

Figure 3 displays cell viability expressed as a relative percentage to the untreated control cells. After exposure to $A\beta_{1-42}$ alone, viability decreased by about 55%, compared to the control. A significant increase ($p<0.05$) in cell viability was observed at all concentrations tested, ranging from 7–12%. It is remarkable that similar cell viability values are obtained for the whole range of concentrations tested, which means there is no dose-dependent pattern. This implies that the same protective effect is achieved

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through a concentration of 1 µM, which is similar to the concentrations obtained after food consumption (2 µM).[9, 34, 35]

Effects of PCA on αS fibril formation

To evaluate the effect of PCA on αS fibril formation, samples of αS alone (70 µM), and in the presence of PCA at different concentrations (10, 20, 50 and 100 µM), were assessed by ThT assay, for a period of 6 days (Fig. 4). ThT fluorescence measurements indicated that PCA inhibited the aggregation of αS in a dose-dependent manner (Fig. 4A). This result was confirmed by TEM (Fig. 5B to 5F). In addition, electron microscope images showed that the high concentration not only affected the extent of fibrillation, but also the morphology of αS fibrils. When αS was incubated alone, the fibrils were longer and compact. Conversely, thin, short fragments were observed following co-incubation with 100 µM of PCA (Figure 5F).

Similarly, electrophoresis of these samples indicated an increase in monomeric forms when αS was incubated with 100 µM of PCA (Figure 5G).

Effects of PCA on pre-formed αS fibrils

Given that PCA was an effective inhibitor of αS fibrillation, we investigated whether it could also reverse fibrillation. 70 µM of pre-formed fibrils (fαS) were incubated at 37°C, without agitation, in the presence of 10, 20, 50 and 100 µM of PCA, for a 6-day period. PCA interacted directly with the protein, and a decrease in ThT fluorescence values was recorded from the first measurements taken (Fig. 6A). Just as in the inhibition of fibrillation assay, lower concentrations, 20 and 50 µM, achieved a very

similar effect. In contrast, at a concentration of 100 µM, PCA is capable of destabilizing around 80% of fαS.

The TEM images support the results described above. For fαS incubated alone, aggregates with a fibrillar structure were observed (Figure 5B). The incubation of αS with 10 µM of PCA resulted in a minor number of aggregates, and they were more scattered (Figure 5C). For intermediate concentrations (20 and 50 µM of PCA), we detected small, thin aggregates, with a characteristic spherical form (Figures D and E). In contrast, no fibrillar structures and very few small aggregates were observed at 100 µM (Figure 5F).

Effects of PCA against different forms of αS-induced toxicity

Figure 6 displays cell viability expressed as a relative percentage of the untreated control cells. Incubation of αS (7 µM) for 2 days, which leads to the formation of oligomers and protofibrils, produces a decrease in cell viability of around 17%, compared with the control (Figure 6). When PCA was co-incubated with αS at different concentrations (1, 5, 25 and 50 µM), a significant increase ($p<0.05$) in cell viability was observed, ranging from 6–33%, in a dose-dependent manner.

After 6 days of exposure to αS, cell viability decreased by around 33%. However, PCA co-incubation significantly increased ($p<0.05$) cell viability at a concentration of 5µM (7–13% increase).

PCA substantially reduced the toxic effects of αS, with this protection being more effective for oligomers and protofibrils than for fibrils.

Discussion

214.

The abnormal assembly of proteins Aβ and αS is responsible for the formation of insoluble aggregates that are related to the origins of AD and PD. Therefore, inhibiting aggregation and destabilizing the fibrils formed are both strategies for tackling these disorders.[26–29] In this study, the inhibitory effects of PCA against Aβ and αS were investigated.

PCA is a metabolite of anthocyanins that can reach the brain.[36] The results obtained show that PCA is a potent inhibitor of aggregation and destabilizer of Aβ and αS fibrils, as confirmed by the ThT fluorescence assay, TEM, electrophoresis and Western blot analysis. In particular, a 100 μM concentration of PCA exerts a significant inhibitory effect (around 80% of activity) for both proteins. These results are in accordance with the literature. PCA presents a high inhibitory effect on αS aggregation and on the destabilization of fαS (from 70–80%). Ardah et al.[37] report that PCA inhibits αS aggregation (60% inhibition) at a ratio of 1:1, measured by ThT assay data, which is in accordance with our work. Destabilization of pre-formed fibrils has not been reported previously, as far as we know, but our results show that the effect of PCA is deserving of attention, because it can contribute to the overall neuroprotective mechanism of this compound. In addition, PCA acted on Aβ and αS pre-formed fibrils at the beginning of the assays, which is important, as PCA is metabolized within 2 hours.[10] Indeed, after this time, about 30% of fibrils had been disaggregated (Figure 2A and 5A).

In comparison to other polyphenols, PCA presents similar percentages in relation to inhibiting $A\beta_{1-42}$ aggregation and destabilizing pre-formed fibrils as others that have been proposed as potent inhibitors, such as resveratrol, (+)-catechin and curcumin, in the same doses (from 80–90%, at 100 μM).[38] Concerning the effects attributed to

anthocyanins and derivatives, it has been demonstrated that cyanidin 3-glucoside prevents the formation of $A\beta_{25-35}$ oligomers and fibril structures, presenting 60% inhibition at 50 μM, a result similar to our findings (63% inhibition at 50 μM of PCA).[39] However, the bioavailability of cyanidin 3-glucoside is somewhat limited, while PCA is assessed in biological fluids following the consumption of anthocyanidins.[10]

PCA plasma concentrations after gastrointestinal digestion and microbiota degradation ranged from 0.2–2 μM, following administration of 500 mg of cyanidin 3-glucoside.[9, 34] Furthermore, it has been reported that PCA itself is present in rat brain following oral administration of Danshen extract, which demonstrates that it can cross the blood-brain barrier (BBB).[36] A major advantage of PCA is that this compound is detected as a consequence of the metabolism of anthocyanins consumed. The implication of PCA pharmacokinetic data is that it can reach the precise place where the action has to be exerted, which reveals it has high potential as a bioactive substance.

Our results show that PCA is also effective against Aβ and αS toxicity. PCA possesses the ability to prevent PC12 cell death caused by $A\beta_{1-42}$ toxicity, resulting in an increase of 12% in living cells, at the minimum concentration tested (1 μM). Other authors have reported similar effects of PCA against $A\beta_{25-35}$-induced toxicity, and obtained an increase in cell viability of between 10 and 15% (1–10 μM concentration).[40] Furthermore, cyanidin 3-glucoside increases cell viability in SH-SY5Y cells by about 11%, at 50 μM.[39] This protection is achieved with 1 μM of PCA, which implies it is more powerful and that it has greater biological significance.

216.

Regarding protection against αS, the literature reports that PCA prevents cell death by MPP+, a toxin involved in PD.[24, 25] Our findings demonstrated that PCA prevents cell death caused by αS oligomers and αS fibrils, and that this effect is stronger for oligomeric forms. This is in contrast to the higher protection against fibril toxicity (50%) reported for gallic acid, which has displayed a 20% increase in cell viability for oligomers.[37]

In conclusion, in this study, it was demonstrated that PCA can inhibit $A\beta_{1-42}$ and αS aggregation and that it destabilizes pre-formed fibrils. Furthermore, PCA protects PC12 cells against Aβ- and αS-induced toxicity. These effects reveal that PCA is a bioactive substance that can tackle neurodegenerative disorders, such as AD and PD. Further studies are required to gain insight into the mechanism of its action.

Abbreviations

PCA: protocatechuic acid

AD: Alzheimer's Disease

PD: Parkinson's Disease

Aβ: Amyloid-β peptide

αS: α-Synuclein protein

fAβ: pre-formed fibrils of Amyloid-β peptide

fαS: pre-formed fibrils of α-Synuclein protein

ThT: Thioflavin T

MTT: Thiazolyl Blue Tetrazolium Bromide

Acknowledgments

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224.

TOC GRAPHIC

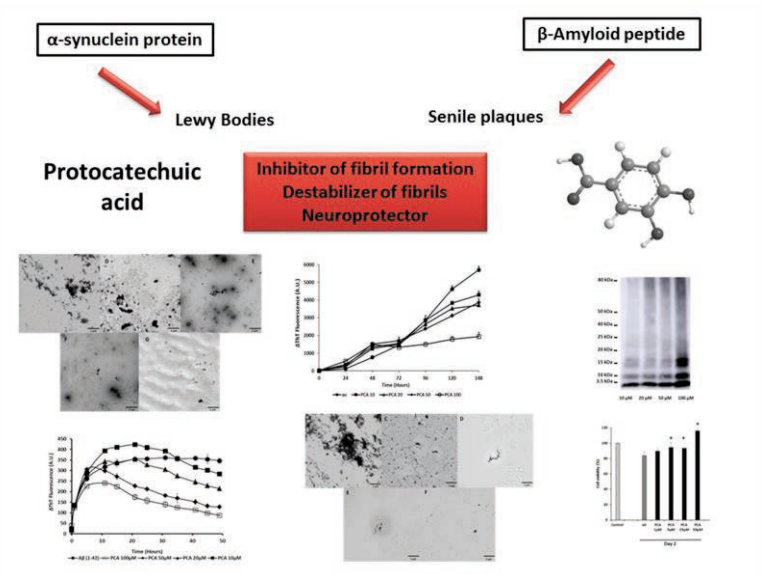
α-synuclein protein
β-Amyloid peptide
Lewy Bodies
Senile plaques
Protocatechuic acid
Inhibitor of fibril formation
Destabilizer of fibrils
Neuroprotector

Figure 1

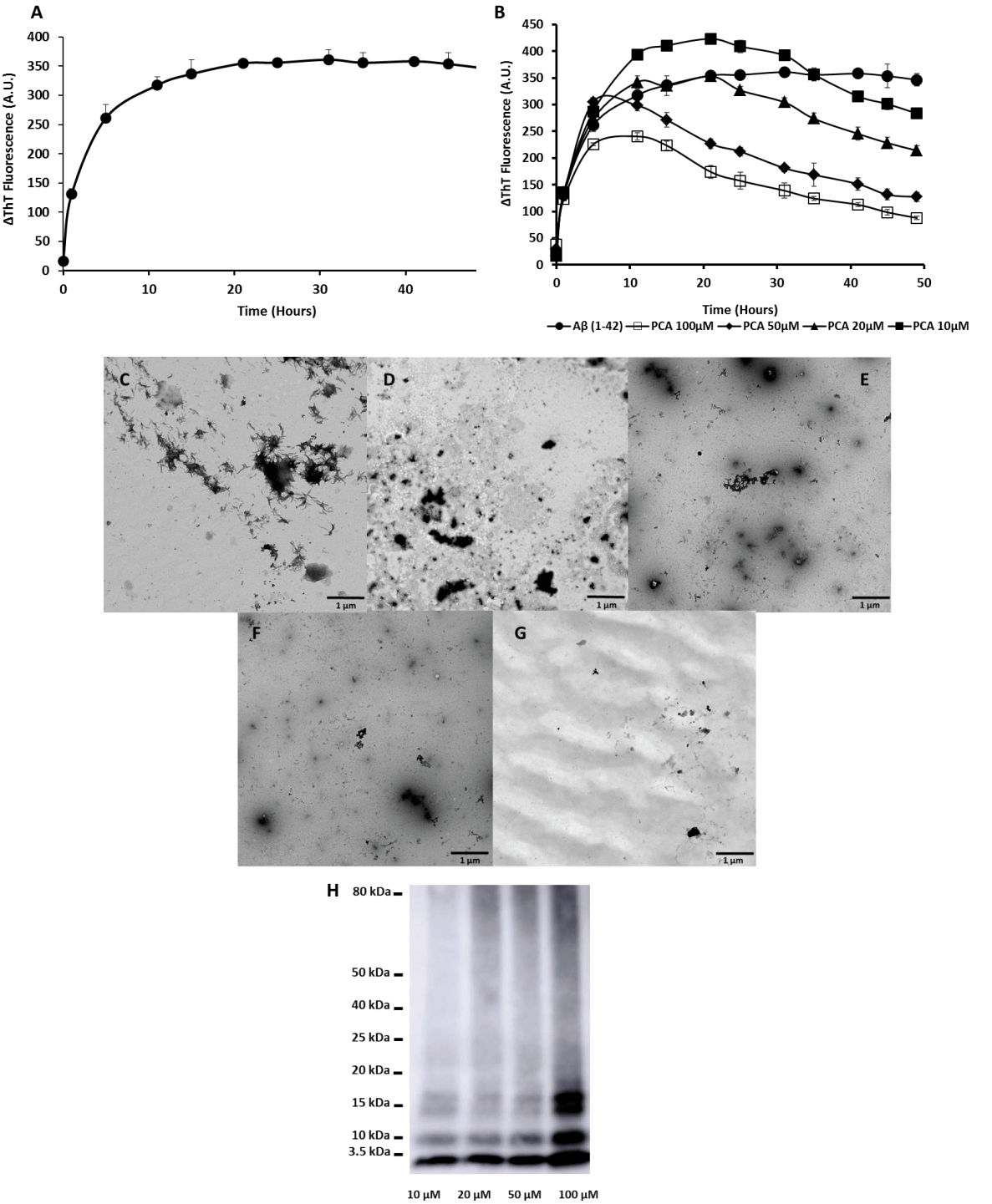
A
ΔThT Fluorescence (A.U.)
Time (Hours)
B
ΔThT Fluorescence (A.U.)
Time (Hours)
Aβ (1-42)
PCA 100μM
PCA 50μM
PCA 20μM
PCA 10μM
C
D
E
F
G
1 μm
H
80 kDa
50 kDa
40 kDa
25 kDa
20 kDa
15 kDa
10 kDa
3.5 kDa
10 μM
20 μM
50 μM
100 μM

Figure 1. PCA inhibits Aβ fibril formation, in a concentration-dependent manner (A) Kinetics of the $A\beta_{1-42}$ aggregation (10 µM of $A\beta_{1-42}$, 25 mM of phosphate buffer, at pH 7.4.) measured by ThT fluorescence at λEx: 450 and λEm: 485, for 48 hours, at 37°C, and with continuous agitation. **(B)** Effects on the kinetics of the $A\beta_{1-42}$ aggregation of PCA, measured by ThT fluorescence. Reaction mixtures containing 10 µM of $A\beta_{1-42}$, 25 mM of phosphate buffer, at pH 7.4, and 10, 20, 50 and 100 µM of PCA. **(C, D, E, F, G)** TEM images of $A\beta_{1-42}$ after 48 hours of incubation with PCA, at different concentrations. The samples were stained using 0.5% uranyl acetate. (C) $A\beta_{1-42}$ alone; (D) $A\beta_{1-42}$ in presence of 10 µM of PCA; (E) $A\beta_{1-42}$ in presence of 20 µM of PCA; (F) $A\beta_{1-42}$ in presence of 50 µM of PCA; (G) $A\beta_{1-42}$ in presence of 100 µM of PCA. The scale bar represents 1µm. **(H)** Effects of PCA at different concentrations on $A\beta_{1-42}$ fibril formation, tested by Western blot. Samples were separated with 4–20% Tris–glycine SDS gel, transferred to a PVDF membrane, and probed with 6E10 and 82E1 antibodies overnight. Bound antibodies were detected with anti-mouse IgG, HRP-linked Antibody, for 1 hour, at room temperature.

Figure 2

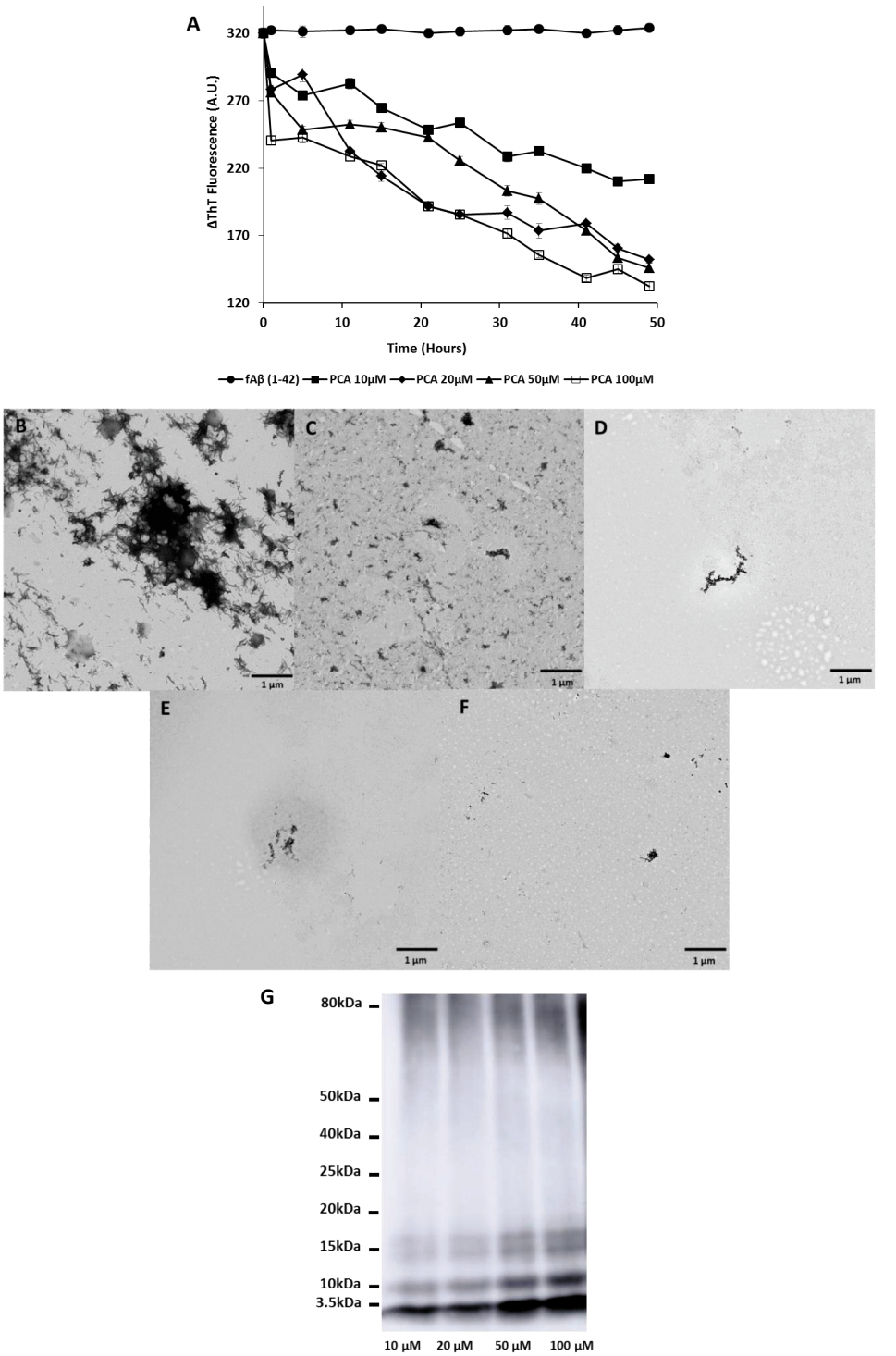
A
320
270
220
170
120
ΔThT Fluorescence (A.U.)
0
10
20
30
40
50
Time (Hours)
fAβ (1-42)
PCA 10μM
PCA 20μM
PCA 50μM
PCA 100μM
B
C
D
1 μm
E
F
G
80kDa
50kDa
40kDa
25kDa
20kDa
15kDa
10kDa
3.5kDa
10 μM
20 μM
50 μM
100 μM

Figure 2. PCA destabilizes pre-formed fibrils of $A\beta_{1-42}$ (fAβ), in a concentration-dependent manner. (A) Effects of PCA on the kinetics of destabilization of fAβ, measured by ThT fluorescence at λEx: 450 and λEm: 485. Reaction mixtures containing 10μM of fAβ, 25 mM of phosphate buffer, at pH 7.4, and 10, 20, 50 and 100 μM of PCA. **(B, C, D, E, F)** TEM images of fAβ after 48 hours of incubation with PCA, at different concentrations. The samples were stained using 0.5% uranyl acetate. (B) fAβ alone; (C) fAβ in presence of 10 μM of PCA; (D) fAβ in presence of 20 μM of PCA; (E) fAβ in presence of 50 μM of PCA; (F) fAβ in presence of 100 μM of PCA. The scale bar represents 1μm. **(G)** Effects of PCA at different concentrations on fAβ destabilization, tested by Western blot. Samples were separated with 4–20% Tris–glycine SDS gel, transferred to a PVDF membrane, and probed with 6E10 and 82E1 antibodies overnight. Bound antibodies were detected with anti-mouse IgG, HRP-linked Antibody, for 1 hour, at room temperature.

Figure 3

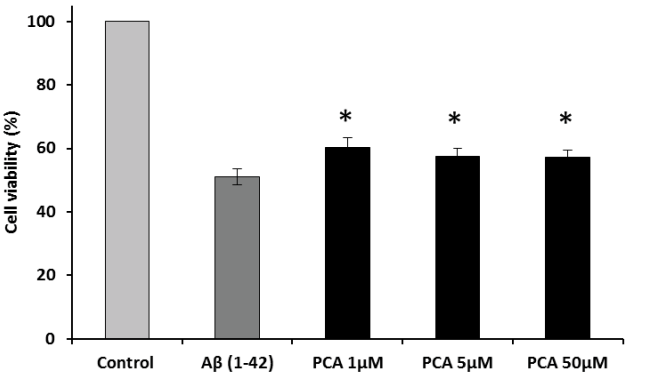

Figure 3. PCA prevents cell death caused by Aβ toxicity. Cell viability (%) (MTT test) PCA at different concentrations (1 / 5 / 50 µM), with 24 hours of pretreatment against Aβ toxicity (5 µM). Results are expressed as mean SEM of four replicates (n = 4). *p<0.05; $A\beta_{1-42}$ versus PCA at different concentrations.

Figure 4

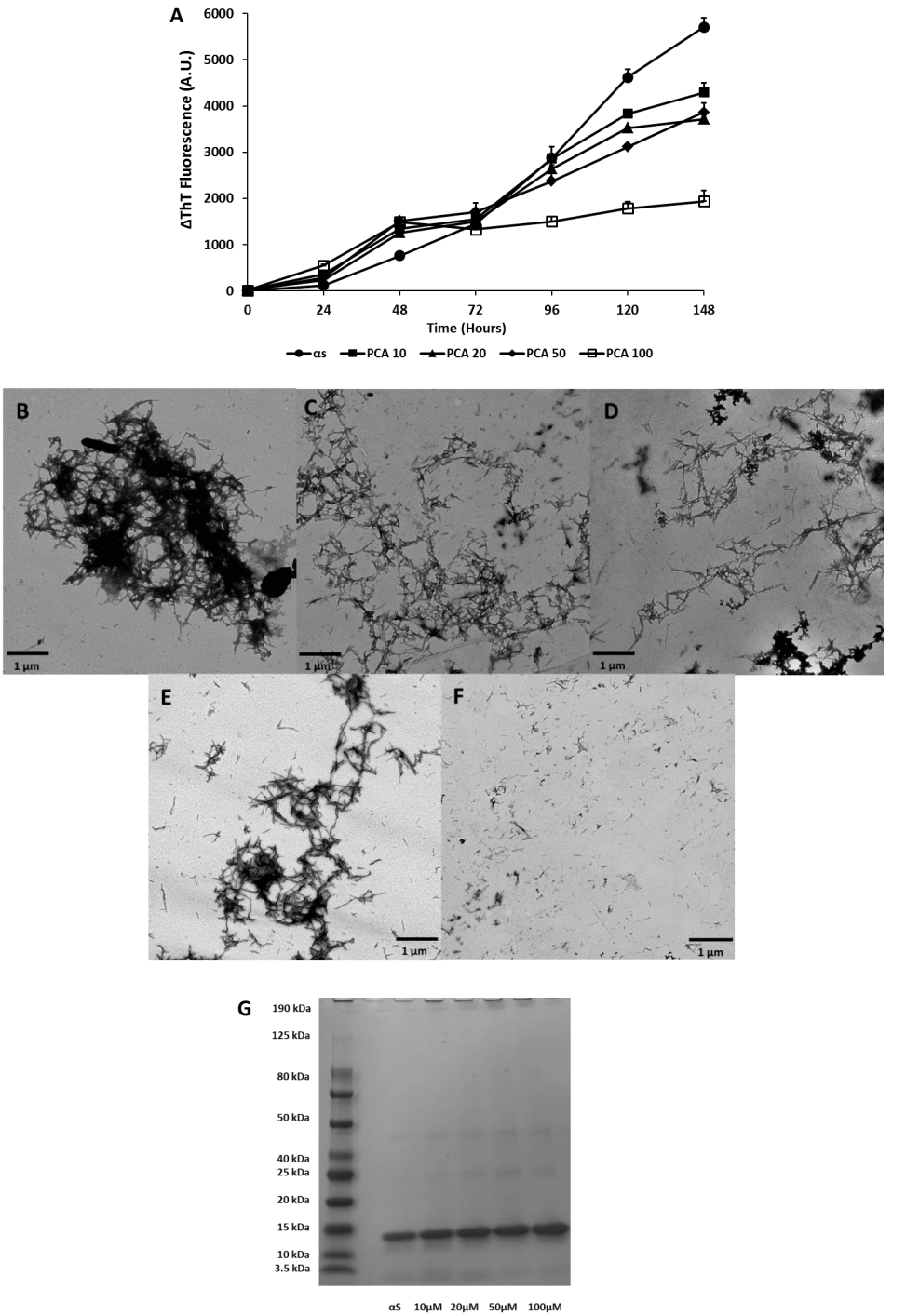
A
ΔThT Fluorescence (A.U.)
6000
5000
4000
3000
2000
1000
0
0
24
48
72
96
120
148
Time (Hours)
αs
PCA 10
PCA 20
PCA 50
PCA 100
B
C
D
E
F
1 µm
G
190 kDa
125 kDa
80 kDa
50 kDa
40 kDa
25 kDa
20 kDa
15 kDa
10 kDa
3.5 kDa
αS
10µM
20µM
50µM
100µM

Figure 4. PCA inhibits αS fibril formation (A) Kinetics of αS aggregation measured by ThT fluorescence at λEx: 450 λEm: 485, for 150 hours, at 37°C, and with continuous agitation. Reaction mixtures contained 70 µM of αS and 25 mM of phosphate buffer, at pH 7.4. **(B)** Effects on the kinetics of αS polymerization of PCA, measured by ThT fluorescence at 450 nm for excitation and 485 nm for emission. Reaction mixtures contained 70 µM of αS, 25 mM of phosphate buffer, at pH 7.4, and 10, 20, 50 and 100 µM of PCA. **(C, D, E, F, G)** TEM images of αS after incubation for 150 hours with PCA, at different concentrations. The samples were stained using 0.5% uranyl acetate. (C) αS alone; (D) αS in presence of 10 µM of PCA; (E) αS in presence of 20 µM of PCA; (F) αS in presence of 50 µM of PCA; (G) αS in presence of 100 µM of PCA. The scale bar represents 1µm. **(H)** Effects of PCA at different concentrations on αS fibril formation, tested by electrophoresis. Samples were separated with 4–20% Tris–glycine SDS gel and stained with Coomassie Blue (0.1% Coomassie R250, 10% acetic acid, 40% methanol).

Figure 5

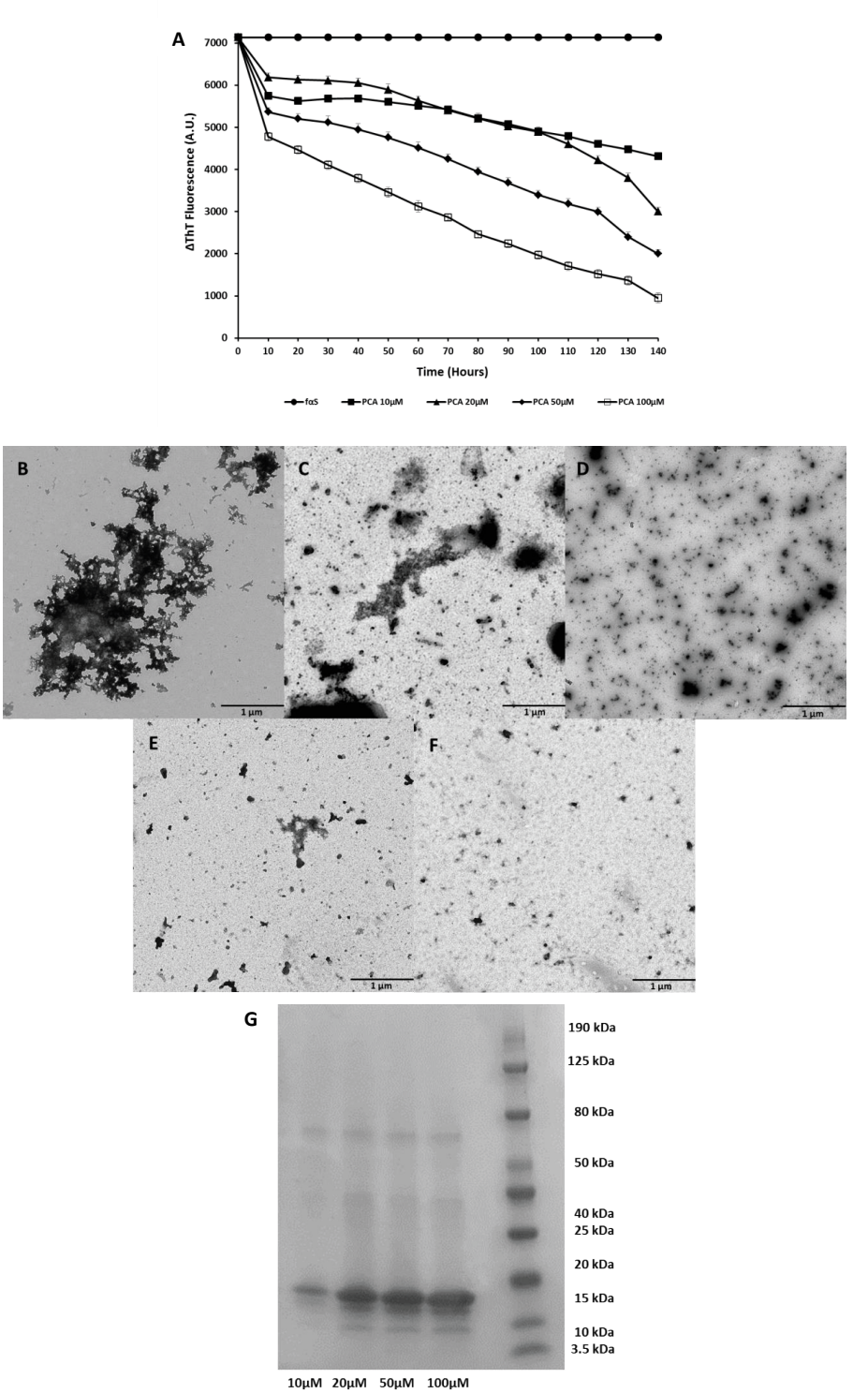
A
ΔThT Fluorescence (A.U.)
Time (Hours)
fαS
PCA 10μM
PCA 20μM
PCA 50μM
PCA 100μM
B
C
D
E
F
1 μm
G
190 kDa
125 kDa
80 kDa
50 kDa
40 kDa
25 kDa
20 kDa
15 kDa
10 kDa
3.5 kDa
10μM 20μM 50μM 100μM

Figure 5. PCA destabilizes αS fibrils (A) Effects of PCA on the kinetics of fαS destabilization, measured by ThT fluorescence at λEx: 450 λEm: 485. Reaction mixtures contained 70 µM of fαS, 25 mM of phosphate buffer, at pH 7.4, and 10, 20, 50 and 100 µM of PCA. **(C, D, E, F, G)** TEM images of fαS after incubation for 150 hours with PCA, in different concentrations. The samples were stained using 0.5% uranyl acetate. (C) fαS alone; (D) fαS in presence of 10 µM of PCA; (E) fαS in presence of 20 µM of PCA; (F) fαS in presence of 50 µM of PCA; (G) fαS in presence of 100 µM of PCA. The scale bar represents 1µm. **(H)** Effects of PCA at different concentrations on fαS fibril destabilization, tested by electrophoresis. Samples were separated with 4–20% Tris–glycine SDS gel and stained with Coomassie Blue (0.1% Coomassie R250, 10% acetic acid, 40% methanol).

Figure 6

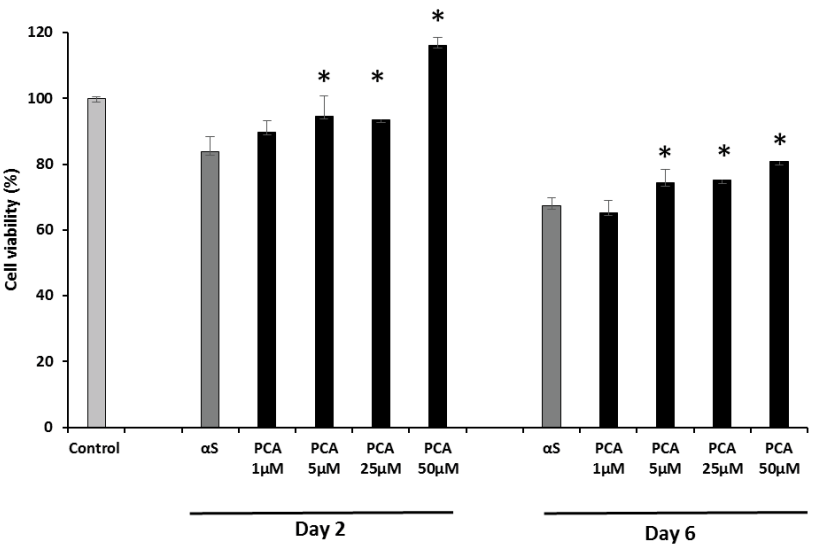

Figure 6: PCA prevents cell death caused by αS toxicity. Cell viability (%) (MTT test), PCA at different concentrations (1, 5, 25 and 50 µM) with pretreatment for 2 and 6 days against αS toxicity (7 µM). Results are expressed as mean SEM of four replicates (n=4). *p<0.05; $A\beta_{1-42}$ versus PCA at different concentrations.

Capítulo 5

Melatonina y otros compuestos indólicos relacionados: inhibición de la formación y desestabilización de fibras de β-amiloide, prevención de la muerte neuronal y posible mecanismos de acción.

Serotonin inhibits amyloid β fibril formation, destabilizes pre-formed fibrils, prevents neuronal death in vitro and possible mechanism of action.

Ruth Hornedo Ortega, Gregory Da Costa, Ana Belén Cerezo, Ana Mª Troncoso, Tristan Richard and Mª Carmen García Parrilla

En preparación para enviar a
Molecular Nutrition & Food Research

Serotonin inhibits amyloid β fibril formation, destabilizes pre-formed fibrils and prevents neuronal death *in vitro*

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Abbreviations: 3IA, 3-indoleacetic acid; **Aβ**, Amyloid-β peptide; **AD**, Alzheimer´s Disease; **CD**, Circular Dichroism; **Mel**, melatonin; **MTT**, Thiazolyl Blue Tetrazolium; Bromide; **Nser**, N-acetyl-Serotonin; **Phol**, tryptophol; **Ser**, Serotonin**; Tee**, tryptophan ethyl ester**; TEM**, Transmission Electron Microscopy**; ThT**, Thioflavin T; **Trpa**, tryptamine; **Tryp**, tryptophan; **TOCSY**, two-dimensional total correlation spectroscopy; **NOESY**, nuclear Overhauser effect spectroscopy; **Kd**, Dissociation constants; **ROS**, reactive oxygen species.

Keywords: amyloid-β, fibril formation, indolic, melatonin, neuroprotection

Abstract

Scope: Amyloid-β peptide (Aβ) is the main component of senile plaques in Alzheimer´s disease (AD). This protein presents neurotoxic properties and the transition from the monomeric form to the aggregated state is a key event in the pathogenesis of this disease. Melatonin is a neurohormone derived from the amino acid tryptophan in a process involving the synthesis of serotonin and other intermediates. In this work, the protective effects against Aβ aggregation and cytotoxicity of eight intermediates in melatonin biosynthesis were evaluated.

Methods and results: A variety of techniques ranging from Thioflavin T (ThT) spectroscopic assay, Transmission Electron Microscopy (TEM), western blot, HPLC, Circular Dichroism (CD) to NMR and cell viability (MTT assay). Serotonin was the compound that presents the most active effect to inhibit Aβ aggregation. Moreover, Serotonin is able to destabilize the preformed fibrils in a dose dependent manner. Additionally, images obtain by TEM and Western Blot analysis showed monomers, dimers and other low molecular weight forms of Aβ when the protein was incubated with serotonin. Moreover, the majority of the indolic compounds tested prevent the Aβ-induced toxicity, being the increase in cell viability between 9-25%. Melatonin and Serotonin were the more actives.

Conclusion: Melatonin and other indolic related compounds present in food show an inhibitory and destabilizer effect on the Aβ fibrils formation and neuroprotective properties.

1. Introduction

AD is the most common neurodegenerative disorder defined by a progressive decline in memoire and other cognitive functions [1]. This disease is characterized by the deposition in the brain of extracellular Aβ leading to the formation of amyloid plaques and by intracellular hyperphosphorylation of τ protein forming neurofibrillary tangles [2]. Currently, the aggregation of Aβ into toxic protofibrils is considered the main key pathogenic event in the onset of AD [3]. For this reason the strategy of inhibiting Aβ aggregation appears to be a valid disease-modifying therapy for AD [4].

Melatonin is a neurohormone secreted by the pineal gland. It is synthesized from the essential dietary amino acid tryptophan through the serotonin pathway (Fig. 1). This molecule is involved in many physiological processes, such as circadian rhythm, antioxidant and the immune system regulation [5, 6]. Concentrations of melatonin vary according to age. With the process of aging a decline of several precursors of melatonin including tryptophan and serotonin occurs and their reduction may be linked to AD appearance [7, 8]. Recently, the neuroprotective effect of melatonin against Aβ aggregation has been demonstrated [9]. Moreover, other work suggests a possible role of the indole ring in the inhibitory properties of indole derivatives [10].

Indolic compounds are present in significant quantities in food. Recently a revision work has reported their concentrations in fermented foods [11]. The appearance of these compounds was related with yeast metabolism [12]. Indeed tryptophol is presents in beer in concentration of 0.242 mg/L [13] and serotonin has been determined in fermented products such as wines and beer in higher concentrations ranging between 2.94-24.2 mg/L [14, 15].

However, other compounds involved in the synthesis of melatonin from tryptophan have not been explored so far.

This study was focused on the protective properties against Aβ aggregation and toxicity of melatonin and other indolic related compounds involved in its synthesis. For this purpose we have used different techniques: ThT essay, cell viability (MTT assay), western blot, TEM, CD, HPLC, NMR.

2. Material and methods

2.1. Chemicals and standards

Tryptophan (Tryp), Tryptamine (Trpa), Serotonin (Ser), tryptophol (Phol), N-acetyl-Serotonin (Nser), Melatonin (Mel), tryptophan ethyl ester (Tee), ThT, DMSO, DMEM-Glutamax, trypsine-EDTA, thiazolyl blue tetrazolium bromide (MTT), PBS, L-glutamine, fetal horse and bovine serum, streptomycin and Tween 20 were purchased from Sigma Aldrich, Steinheim, Germany. 3-indoleacetic acid (3IA) and uranyl acetate dihydrate was purchased from Fluka Biochemika, Steinheim, Germany. $A\beta_{1-42}$ peptide was provided by Alexotech, Umeå, Sweden. Na_2HPO_4/NaH_2PO_4 and NaCl were supplied by Panreac, Castellar del Vallès, Barcelona, Spain. PC12 cells were supplied by ATCC, Manassas, USA.

Aβ N-terminal specific antibody 82E1 was obtained from IBL, Minneapolis, Minnesota. Aβ (1-16) monoclonal antibody 6E10 was purchased from Covance, Japan. Anti-mouse IgG, HRP-linked antibody was supplied by Cell Signaling Technology, Danvers, Massachusetts. HyperPAGE prestained protein marker was purchased from Bioline, London, UK. L-tryptophan, SDS-Solution 10%, glycine, bromophenol blue and glycerol anhydrous were purchased from Applichem, Darmstadt, Deutschland. 2-Mercaptoetanol, 10× Tris/glycine/SDS (10× premixed electrophoresis buffer contains 25 mM Tris, 192 mM glycine, 0.1% SDS, pH 8.3); 10× Tris/glycine (10× premixed electrophoresis buffer), 4-20% Mini-PROTEAN® TGX Stain-Free gel, 15 well precast polyacrylamide gel (8.6 × 6.7 cm) and Immune-Blot PVDF membrane were purchased from Bio-Rad, München, Germany. ECL 2 Plus western blotting substrate was

purchased from Thermo Scientific, Rockford, USA. Carbon-coated grids (300 mesh, copper) were purchased from EMS, Hatfield, Pennsylvania, United States.

2.2. Measurement of Aβ aggregation and destabilization using ThT assay

The process of aggregation was monitored using the ThT assay. ThT is used as a dye to monitor the formation of amyloid aggregates [16].

Stock solution of 221 µM of $A\beta_{1-42}$ protein and 1 mM were prepared in 25 mM Na_2HPO_4/NaH_2PO_4 buffer (25 mM) pH 7.4, 140 mM NaCl. A stock solution of each compound was prepared at 50 mM in DMSO.

Concerning aggregation assay, $A\beta_{1-42}$ (10 µM final concentration) was mixed in equal volume with each compound (100 µM final concentration) and ThT (25 µM final concentration) on black 96-well plates. ThT fluorescence was monitored using a multi-detector microplate reader (Synergy HT, Biotek®) fluorescence spectrophotometer set at 450 nm for excitation and 485 nm for emission wavelengths and fluorescence emission data was recorded each 2 h during a 48 h period of time.

The same protocol was used for disaggregation assay, with the only difference that the $A\beta_{1-42}$ protein was previously fibrillated during 3 days at 37ºC with continuous agitation (400 rpm).

At the end of the experiment, samples were frozen at -80ºC. These samples were used later for electrophoresis and western blot purposes and TEM analysis.

2.3. Sedimentation assay

Stock solution of 1 mM $A\beta_{1-42}$ was prepared by solubilizing the lyophilized peptide upon brief vortexing in sterile water at 4°C, followed by sonication for 1 minute. The stock solution was aliquoted and stored at – 20ºC. All subsequent steps were carried out at 4ºC to prevent any Aβ peptide polymerization. Serotonin was solubilized in ethanol (1 mg/mL stock solution), then diluted to reach either a 100 µM final concentration. Aliquots were stored at – 20 °C and added to a solution of phosphate buffer (10 mM final concentration, pH 7.2) and Aβ peptide (100 µM final concentration). Typical experiment was carried out on a reaction mixture containing 80 µL of the phosphate buffer solution, 10 µL of the Aβ peptide solution, and 10 µL of ethanol without or with serotonin; sonication was performed for 5 min to avoid peptide aggregation as much as possible at initial time. The mixture was incubated at 15ºC for 3 days. The solution was a centrifuged at 85000 rpm for 30 min at 15ºC. The pelleted fraction enriched in Aβ fibrils was discarded and the supernatant was analyzed by HPLC using an UV detection at 220 nm.

2.3. Electrophoresis and western blot analysis

15 µL of samples (after inhibition and destabilization assays) were diluted with 5 µL of Tris-Base (0.125 M), glycerol (20%, v/v), SDS (4%), 2-β-mercaptoetanol (10%, v/v) and bromophenol blue (0.06%, w/v) buffer. After gel electrophoresis (4-20% precast polyacrylamide) proteins were transferred to PVDF membranes by applying 310 mA for at least 1 hour. The membrane was boiled during 5 min with PBS and then rapidly cooled. The membrane was incubated with a mix of antibodies (6E10 and 82E1. 1/6000 dilution) overnight at 4ºC. Afterwards, the samples were cleaned with TBST and incubated with anti-mouse IgG antibody (1/10000 dilution) for 1 hour. Later the

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membrane was cleaned with TBST and examined by chemiluminescence (Amersham Imager 600, GE Healthcare Life Sciences).

2.4. TEM images

10 µL of samples (after inhibition and destabilization assays) were placed on a 300 mesh carbon-coated grid and incubated 5 min. Then the excess of fluid was removed and the grids were negatively stained for 1.5 min with 5 µL of a 0.5% uranyl acetate solution. Excess of fluid was removed and the samples were viewed using a Zeiss Libra 120 transmission electron microscope operating at 80 kV.

2.5. CD analysis

CD spectra were acquired at 20ºC, under N_2 atmosphere, in a quartz cell with a path length of 0.2 cm on a JASCO J-815 spectropolarimeter, equipped with a Peltier device for temperature control. Near-UV-CD spectra were measured between 200 and 250 nm with a scanning speed of 50 nm/min. Data collection was repeated five times scanning from 250 to 190 nm. Solutions of $A\beta_{1-42}$ and $A\beta_{1-42}$ with serotonin were prepared according the protocol described previously in sedimentation assay.

2.6. NMR analysis

NMR samples were prepared by dissolving the $A\beta_{1-42}$ in 500 µL of DMSO d6 (0.5 mM final concentration). Serotonin was dissolved in 300 µL of DMSO d6 at a 60 mM concentration. Titration experiments were performed by addition of small amounts of serotonin to the peptide solution. All 1D and 2D NMR spectra were recorded on a Bruker Avance 600 MHz spectrometer, and calibrated using TSP-d4 as an internal standard for proton chemical shifts. NMR experiments were recorded at 300 K, and

data were processed using TOPSPIN software (Bruker Topspin). The sequence-specific assignment of the $A\beta_{1-42}$ was obtained using two-dimensional total correlation spectroscopy (TOCSY) and nuclear Overhauser effect spectroscopy (NOESY) experiments.

2.5. PC12 cell culture and MTT assay

Rat pheochromocytoma cells (PC12) were obtained from the American Type Culture Collection (ATCC, Manassas, USA). They were maintained in 75 cm^3 culture flasks containing 20 mL of DMEM Glutamax supplemented with 100 units/mL penicillin, 100 mg/mL streptomycin, 15% fetal horse serum and 2.5% fetal bovine serum at 37ºC with 5% CO_2. Cells were passaged every 3-4 days and detached from flasks using trypsin-EDTA. Cells counts were performed using trypan blue to stain non-viable cells.

$A\beta_{1-42}$ (5 µM final concentration) and indolic compounds (50 µM final concentration) were diluted with serum-free culture medium and incubated with cells (at a density of 30000 cells/well) for 24 h (without pretreatment modality) or incubated during 24 h on a thermoblock with continuous agitation and then exposed to PC12 cells for 24 h(pre-treatment modality) to screen the neuroprotective effect of compounds. Cell viability was determined by using the MTT reduction assay. Cells were treated with 0.5 mg/mL MTT for 3 h at 37 °C. The dark blue formazan crystals formed in intact cells were solubilized with DMSO for half-an-hour. The absorbance was measured at 540 nm with a microplate reader (Synergy HT, Biotek). Results were expressed as the cell viability percentage in relation to the viability of control cells (100%). All experiments were repeated at least in triplicate and performed in quadruplicate.

2.6. Statistical analysis.

For cell viability measurements, One-way analysis of variance (ANOVA test) ($p < 0.05$) was used [17].

3. Results

3.1. Inhibitory effects of indolic compounds on $A\beta_{1-42}$ aggregation

Eight indolic compounds were investigated: Tryp, Trpa, Ser, NSer, Phol, 3IA, Mel and Tee. To determine whether indolic compounds inhibit the assembly of Aβ into filaments, ThT assays were used. ThT fluorescence is correlated to β-sheet formation and to fibril formation [16]. As displayed in Fig. 2A, the incubation of $A\beta_{1-42}$ at 37ºC with continuous stirring, showed a rapidly increase on the ThT fluorescence in the first hour of the experiment taking place the typical lag phase of aggregation process in the firsts ten min of the experiment. This observation can be explained due to the use of continuous agitation conditions and the presence of salts. Comparable results were described a lag time of 3 min using similar experimental conditions [18]. The progressive increase of ThT fluorescence during 50 h indicate the formation of β-sheet structures of $A\beta_{1-42}$ being this time sufficient to observe the aggregate formation. This result was confirmed by TEM images (Fig. 2C).

To evaluate the inhibitory capacity of indolic compounds, initial screening was performed. ThT fluorescence assay was used to measure the amount of amyloid fibrils after the addition of a fixed concentration of each compound (100 µM). The screening revealed that only serotonin significantly caused a dramatic reduction of aggregation (Fig. 2A). Serotonin inhibits around 80% on the β-sheet formation at 100 µM. This

result is comparable with the effect on $A\beta_{1-42}$ fibril formation of different polyphenols studied previously such as catechin and curcumin [19]. In addition, the inhibitory effect of serotonin at different concentrations has been examined. $A\beta_{1-42}$ was co-incubated with 10, 50 and 100 µM of serotonin. 50 and 100 µM of serotonin were the active concentrations that disrupted the fibril formation presenting a significant inhibition of 30 and 80% respectively compared with the control. In contrast, at concentration of 10 µM no inhibitory effect was observed (Fig. 2B).

To confirm these results, TEM experiments were used to visualize the morphologies of $A\beta_{1-42}$ in the presence or absence of Serotonin. When $A\beta_{1-42}$ was incubated alone for 50 h aggregates were observed (Fig. 2C), whereas samples containing $A\beta_{1-42}$ with 50 and 100 µM of serotonin were devoid of fibrils and containing only a very small amount of aggregates (Fig. 2D and 2E). Hence, the number of aggregates decreased with increasing concentration of serotonin, dose-dependent effect.

To monitor the efficacy of serotonin to destabilize $A\beta_{1-42}$ fibrils, different concentrations of this compound were added to pre-formed $A\beta_{1-42}$ fibrils and ThT fluorescence was measured during 48 h. Our results have revealed that serotonin destabilizes pre-formed Aβ fibrils in a dose dependent manner (65, 52, and 22%, for 100, 50 and 10 µM, respectively) (Fig. 3A). These results were confirmed by TEM experiments after ThT assays. TEM observation of $A\beta_{1-42}$ pre-formed fibrils shows huge amounts of Aβ fibrils (Fig. 3B). When $A\beta_{1-42}$ pre-formed fibrils were co-incubated with increasing concentrations of serotonin, the number of fibrils decrease (Fig. 3C and 3D).

3.2. Western blot analysis.

In order to investigate whether serotonin could interfere in $A\beta_{1-42}$ fibril formation, samples which proceed of ThT inhibition assays were used for analysis by Western Blot. 6E10 and 82E1 antibodies (reactive to amino acid residue 1-16 of beta amyloid), were used to detect selectively Aβ monomers, oligomers and fibrils with the purpose to assess the size distribution which were present in ThT samples.

Regarding inhibition of aggregation of $A\beta_{1-42}$ experiment, we have analized $A\beta_{1-42}$ alone and $A\beta_{1-42}$ with serotonin at the two actives concentrations (50 and 100 µM). Fig. 2F, showed that when the $A\beta_{1-42}$ was incubated with 100 and 50 µM of serotonin the signals are more intense in the bands corresponding to 3.5-15 kDa (monomers and dimers of Aβ) in comparison with Aβ alone, which is indicative of an inhibition of fibril formation.

3.3. Sedimentation assay

Sedimentation assays were carried to insure that serotonin inhibit the formation of insoluble Aβ aggregates. Aβ incubated alone or Aβ co-incubated with serotonin were analyzed by HPLC. Incubated solutions were previously centrifuged to remove insoluble aggregates and then the supernatants were analyzed by HPLC. Fresh Aβ was eluted, at a retention time of 3.8 min, as a single peak, probably monomers (Fig. 4). After incubation 3 days at 15ºC, the monomer peak disappeared. The chromatogram shows the presence of limited amounts of $A\beta_{1-42}$ in the soluble fractions compared to chromatogram of freshly prepared Aβ because fibrils were spun out by centrifugation. After co-incubation with serotonin, a single peak at retention time of 3.8 min was observed and attributed to soluble Aβ species. This observation confirms that serotonin inhibits the conversion of soluble Aβ into insoluble fibrils.

3.4. CD studies

To examine and compare conformational changes in secondary structure the structural behavior of $A\beta_{1-42}$, with or without serotonin, was monitored using CD spectrometry. Fig. 5A shows the far-UV CD spectra of protein before and after incubation 3 days, with or without serotonin. The freshly prepared Aβ is an unstructured protein with a major negative peak around 200nm characteristic of random coil. With incubation, Aβ alone adopts a β-sheet-structure as shown by negative peaks in the 215-220 nm region which is characteristic for an aggregated protein, as consistent with literature [20, 21]. During co-incubation with serotonin, Aβ remained in random coil after incubation 3 days. Serotonin prevents the conformational transition from random coil to β-sheet-structure during the fibrillation process.

3.5. NMR analysis

The NMR approach was used to obtain data about the specific interaction between $A\beta_{1-42}$ and serotonin. NMR analyses were carried out in DMSO-d_6 to prevent fast Aβ aggregation in the NMR concentration range [22]. To monitor the interaction, titration experiments were performed by addition of small amounts of serotonin to the peptide solution and monitored using NMR spectra [23]. $A\beta_{1-42}$ resonances were attributed by mean of 2D-NMR experiments. The addition of serotonin to a solution of Aβ induced chemical shift variations of peptide resonances (Fig. 5B). Many of the proton signals of $A\beta_{1-42}$ (Val24, Asp7, Asp23 and Tyr10) were shifted to higher field with saturable binding process indicating a direct interaction and a specific binding between $A\beta_{1-42}$ and serotonin (Fig. 5C). Binding constants in solution was determined by fitting the chemical shift changes of amide and aromatic protons whose variations have been

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measured unambiguously without overlapping. Dissociation constants (Kd) were determined by curve fitting with a one-site saturation and nonspecific binding model. The Kd values obtained from the chemical-shift changes of Val24, Asp7, Asp23 and Tyr10 were 1.9 ± 1.2, 5.4 ± 1.9, 0.5 ± 0.2, 4.1 ± 0.6 mM, respectively.

3.6. Neuroprotection against Aβ toxicity in PC12 cells by indolic compounds.

Reduction of MTT was employed as an indicator of cell viability (PC12 cells), which is extensively used as a quantitative and consistent colorimetric assay [24]. First of all, the toxicity on PC12 cells of each compound was monitored; at 50 µM none produce cell death (Fig. 6). Protective effects against Aβ-induced toxicity of the eight indolic compounds were measured. For this objective, we probed two different treatments to establish the protocol to apply. On the one hand, the whole compounds were tested without pretreatment (NPT): $A\beta_{1-42}$ (5 µM final concentration) and compounds (50 µM final concentration) were mixed and incubated directly with cells for 24 h on the other hand with pre-pretreatment (PT): $A\beta_{1-42}$ (5 µM) and compounds (50 µM) were mixed and incubated for 24 h in agitation at 37ºC and then incubated with cells for 24 h. Finally, the cell viability through MTT assay was carried out.

Fig. 6 displays the cell viability expressed as a percentage relative to the untreated control cells. After exposure to $A\beta_{1-42}$ alone, viability decreased around 50% compared with the control. Results show between the two treatments that PT protocol increased significantly ($p<0.05$; $p<0.01$) cell viability for the majority of compounds. Tryp and Tee did not present any neuroprotective effect. They exhibited increase of cell viability in the rank order Mel > Ser > Trpa > NSer > 3IA > Phol. Melatonin is the more active compound followed by serotonin (cell viability 25% and 24%, respectively). In this case

with both protocols a significant increase of cell viability was observed being the higher augmentation with PT than NPT (cell viability 75 and 60%, respectively).

To confirm the protective effect of serotonin at different concentrations (1, 5, 10 and 50 μM) against Aβ-induced toxicity was carried out using PT protocol.

Serotonin presents a dose dependent tendency being the increase of cell viability higher for high concentrations. The augmentation ranged between 4-25% ($p < 0.001$; $p < 0.0001$) (Fig. 7).

4. Discussion

The inhibition of aggregation or the disaggregation of Aβ oligomers and fibrils by bioactive compounds presents in food matrices are greatly studied to the prevention and treatment of AD. In this context, polyphenols such as curcumin, myricetin, morin, quercetin, kaempferol (+)-catechin, (−)-epicatechin, nordihydroguaiaretic acid, tannic acid and stilbenes have been extensively investigated [25-30]. Furthermore, the role of different extracts of plants and foods have also been investigated with satisfactory effects against Aβ toxicity [31-32].

The presence of bioactive compounds in fermented beverages has long been observed and they have been studied with great attention. Yeast transforms certain molecules like amino acids into biologically active compounds. Among these, the case of amino acid tryptophan is of interest, since it is the precursor of at least three biologically active compounds: melatonin, serotonin, and tryptophol [12]. Melatonin appears in fermented products in order of ng-pg/mL. However, the concentrations of tryptophol

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or serotonin are very higher (mg/L). Their occurrence in foods and beverages contribute to a dietetic intake that might exert beneficial effects [11].

Furthermore, very few studies have probed the inhibitory effect of indolic compounds on the Aβ fibrillation process. Melatonin has been the most studied indolic compound and many pharmacological properties have been endorsed. It shows a potent antioxidant activity [34], promotes the synthesis of numerous endogenous antioxidant enzymes [35], and improves mitochondrial energy metabolism [36]. Furthermore, neuroprotective effects have been attributed to melatonin. In this way, melatonin reduces Aβ-induced oxidative stress related to reactive oxygen species (ROS) and proinflamatory cytokines *in vivo* studies [37]. As a result of these effects, melatonin protects brain neurons from damage and death by increasing viability in hippocampal neurons around 14% and glial cells following treatment with $A\beta_{1-40}$, $A\beta_{25-40}$, and $A\beta_{1-28}$ [9]. Our results in accordance with this paper, show an increase of cell viability around 20-25% by melatonin.

Concerning the disruption or the destabilization of Aβ oligomers and fibrils, our work demonstrate that serotonin is a potent inhibitor for both processes (80-65% of effect respectively) by ThT assays and confirmed by TEM and Western blot analysis. These findings show similar effects reported by other polyphenols around 70-80% on the inhibition of Aβ aggregation (resveratrol, piceid and rosmaric acid) [28, 38]. In contrast with our results, have been reported that tryptophol and 3-indoleacetic acid presented inhibitory effect using lysozyme (protein with similar aggregation pattern of Aβ), however under our conditions any effect have been observed. Moreover, and in

accordance with us, tryptophan and melatonin did not provide any effect on the Aβ fibrillation process [9, 10].

In presence of serotonin the decrease of ThT fluorescence began between the 5-15 h of the experiment. In this moment the elongation phase was occurred and consequently oligomers and protofibrils have been formed [39]. In base to other reports, compounds that interfere in the elongation phase but not in the lag phase, can inhibit the protein assembly by blocking the interstrand hydrogen bond formation and not by stabilizing the nonamyloidogenic conformations of the proteins [40]. Accordingly, serotonin may impede $A\beta_{1-42}$ insterstand hydrogen bond formation preventing the fibrillation.

Furthermore, the majority of compounds tested protect to the neuronal death induced by Aβ being the most active melatonin and serotonin. Serotonin increase the cell viability in the first 24 h of Aβ fibrillogenesis (Ser + $A\beta_{1-42}$ during 24 h with agitation (PT)) such as when amyloid oligomers and fibrils were already formed (Ser + $A\beta_{1-42}$ PT + 24 h).

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Figure 1

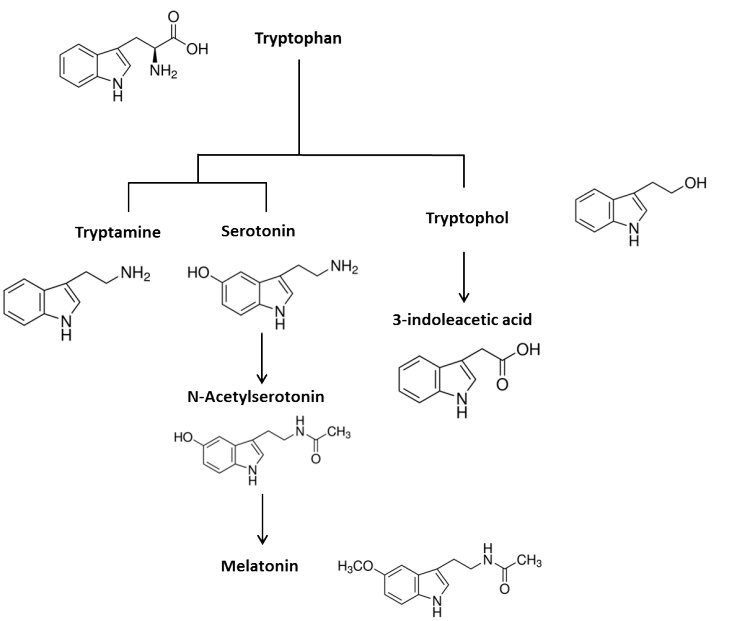
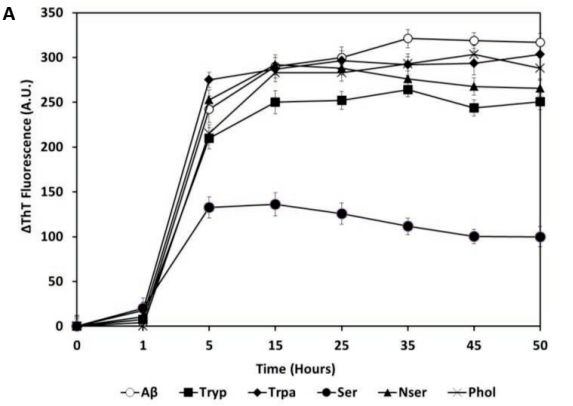
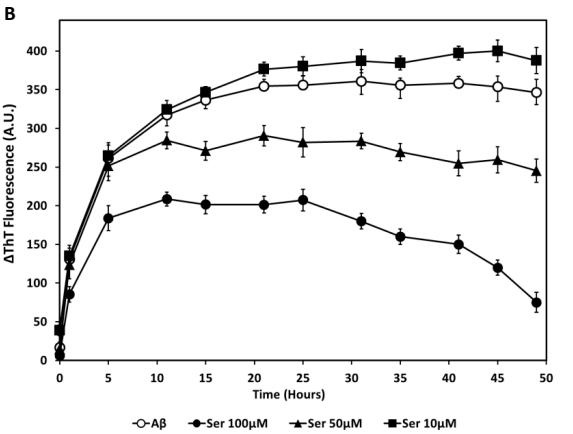
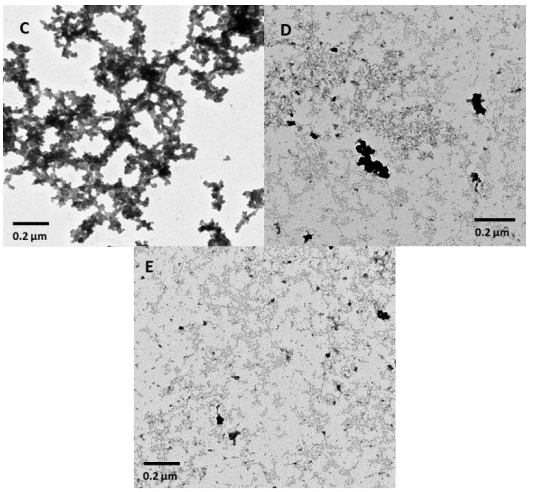


Figure 1. Tryptophan metabolism and intermediates implicated in the synthesis of melatonin.

Figure 2


A
ΔThT Fluorescence (A.U.)
Time (Hours)
Aβ
Tryp
Trpa
Ser
Nser
Phol


B
ΔThT Fluorescence (A.U.)
Time (Hours)
Aβ
Ser 100μM
Ser 50μM
Ser 10μM


C
D
E
0.2 μm
0.2 μm
0.2 μm

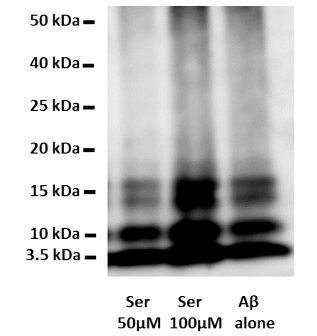
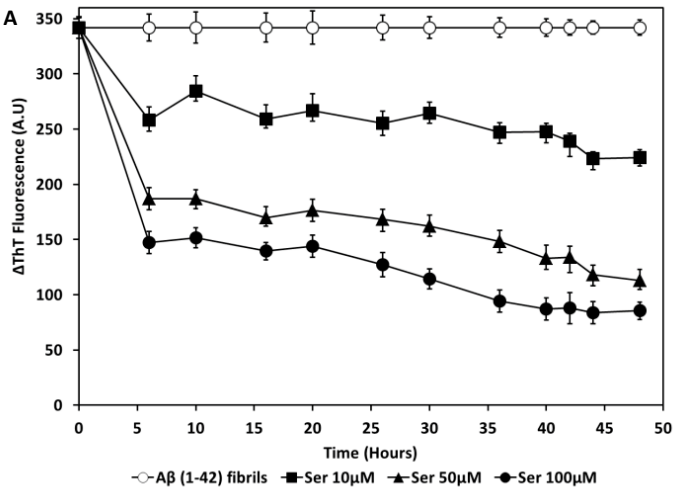

F
50 kDa
40 kDa
25 kDa
20 kDa
15 kDa
10 kDa
3.5 kDa
Ser 50μM
Ser 100μM
Aβ alone

Figure 2. Inhibition of Aβ fibrils by indolic compounds (Tryp, Trpa, Ser, NSer, Phol, 3IA, Mel and Tee). **(A)** kinetics of $A\beta_{1-42}$ (10 µM) polymerization in absence (control) and presence of indolic compounds (100 µM) in 25 mM phosphate buffer (pH 7.4) measured by ThT assays; **(B)** kinetics of $A\beta_{1-42}$ (10 µM) polymerization in presence of Ser (10, 50 and 100 µM) measured by ThT assays; **(C)** TEM observation of $A\beta_{1-42}$ alone (10 µM); **(D)** TEM observation of $A\beta_{1-42}$ in presence of 50 µM Ser; **(E)** TEM observation of $A\beta_{1-42}$ in presence of 100 µM Ser. For TEM experiments, samples were incubated 50 h and stained using 0.5% uranyl acetate. The scale bar represents 0.2 µm; **(F)** Effects of Ser at 50 and 100 µM on the inhibition of Aβ fibrils tested by Western blot. Samples were separated with 4-20% Tris–glycine SDS gel and transferred to a PVDF membrane and probed with 6E10 and 82E1 antibodies overnight. Bound antibodies were detected with anti-mouse IgG, HRP-linked Antibody for 1 h at room temperature.

Figure 3


A
ΔThT Fluorescence (A.U)
Time (Hours)
Aβ (1-42) fibrils
Ser 10μM
Ser 50μM
Ser 100μM

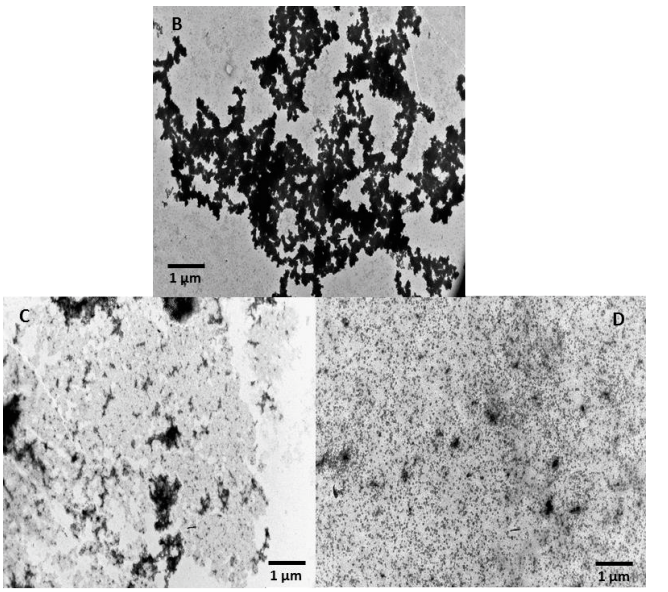

B
1 μm
C
1 μm
D
1 μm

Figure 3. Destabilization of Aβ fibrils by indolic compounds: **(A)** kinetics of pre-formed $A\beta_{1-42}$ (10 µM) destabilization in absence (control) and presence of Ser (10, 50 and 100 µM) in 25 mM phosphate buffer (pH 7.4) measured by ThT assays; **(B)** TEM observation of pre-formed $A\beta_{1-42}$ alone (10 µM); **(C)** TEM observation of $A\beta_{1-42}$ in presence of 50 µM Ser; **(D)** TEM observation of $A\beta_{1-42}$ in presence of 100 µM Ser. For TEM experiments, samples were incubated 50 h and stained using 0.5% uranyl acetate. The scale bar represents 1 µm.

Figure 4

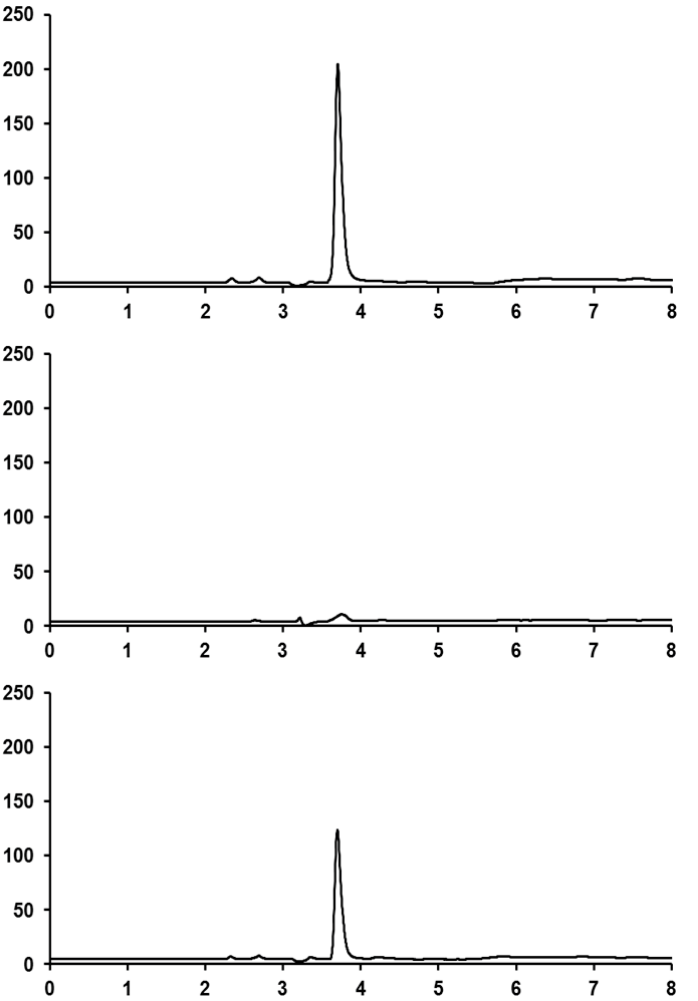


Figure 4. HPLC traces of the supernatants (UV detection at 220 nm), after centrifugation of $A\beta_{1-42}$ in the absence or presence of Ser: $A\beta_{1-42}$ freshly prepared (top chromatogram); $A\beta_{1-42}$ alone incubated 3 days at 15ºC (middle chromatogram); $A\beta_{1-42}$ incubated with Ser 3 days at 15ºC (bottom chromatogram).

Figure 5

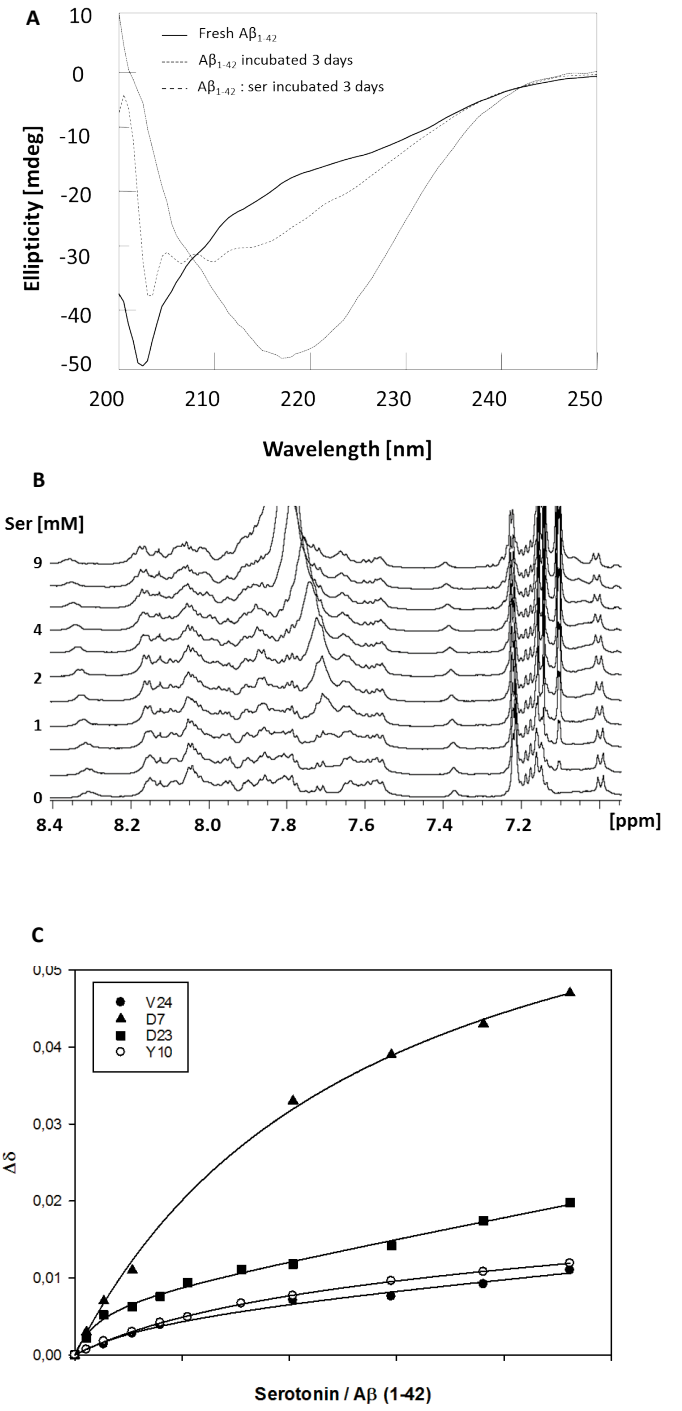

A
Fresh Aβ1-42
Aβ1-42 incubated 3 days
Aβ1-42 : ser incubated 3 days
Ellipticity [mdeg]
10
0
-10
-20
-30
-40
-50
200
210
220
230
240
250
Wavelength [nm]
B
Ser [mM]
9
4
2
1
0
8.4
8.2
8.0
7.8
7.6
7.4
7.2
[ppm]
C
V24
D7
D23
Y10
Δδ
0,05
0,04
0,03
0,02
0,01
0,00
Serotonin / Aβ (1-42)

Figure 5. (A) Far-UV CD spectra of $A\beta_{1-42}$ incubated in the absence or presence of Ser: $A\beta_{1-42}$ freshly prepared (solid line); $A\beta_{1-42}$ alone incubated 3 days at 15ºC (dotted line); $A\beta_{1-42}$ incubated with Ser 3 days at 15ºC (dashed line). **(B)** 1H NMR spectra of the amidic and aromatic protons of $A\beta_{1-42}$ upon Ser addition. **(C)** Chemical shift variations of $A\beta_{1-42}$ protons with increasing Ser/$A\beta_{1-42}$ ratios: solid lines represent the best fit of the experimental data used for Kd determination; black circle, square and triangle are for Val24, Asp7 and Asp23 amide protons, respectively. Open circle, are for Tyr10 aromatic proton.

Figure 6

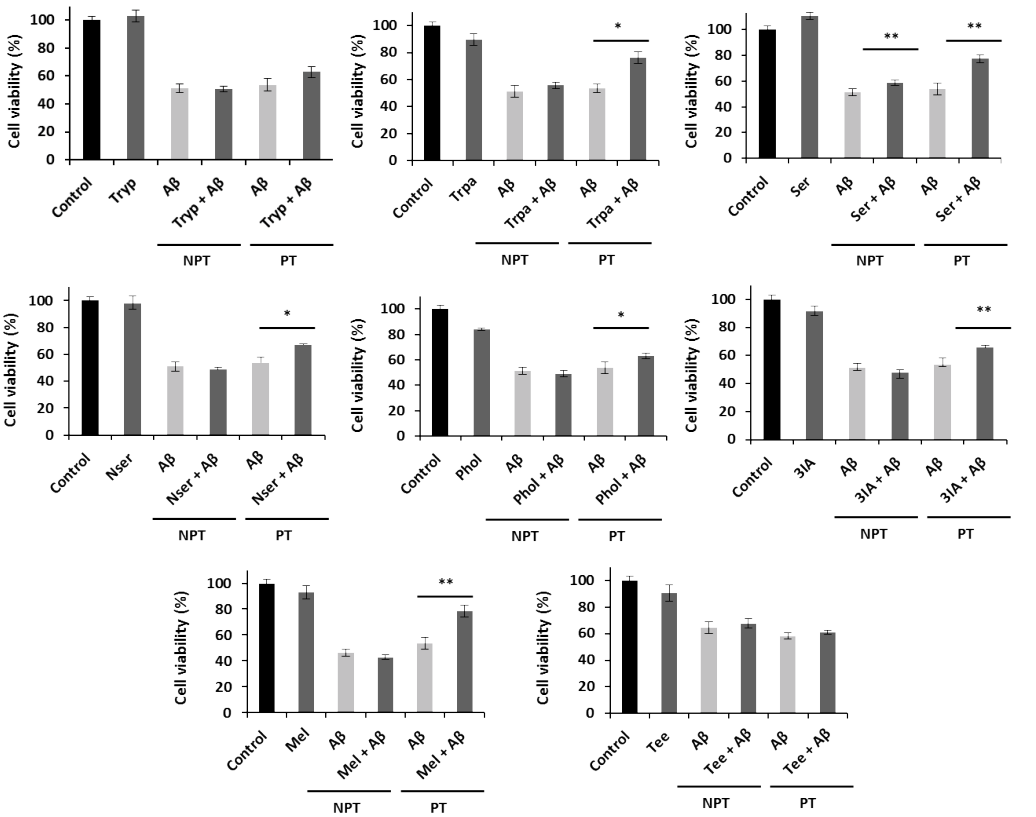


Figure 6: Cell viability (%) (MTT assay) of Tryp, Trpa, Ser, NSer, Phol, 3IA, Mel and Tee (50 µM) without pretreatment (NPT) and with pretreatment of 24 h (PT) against $A\beta_{1-42}$ toxicity (5 µM). Results are expressed as mean SEM of four replicates (n=4). * $p < 0.05$; ** $p < 0.01$ $A\beta_{1-42}$ versus compounds.

Figure 7

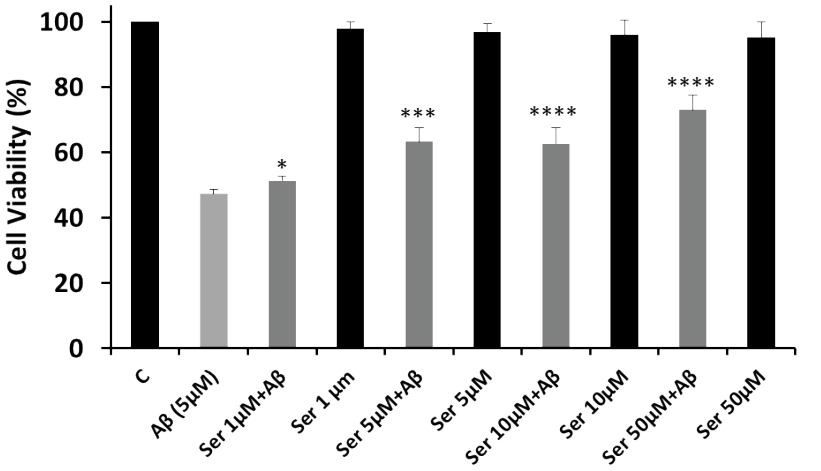


Figure 7: Cell viability (%) (MTT assay) Ser at different concentrations (1/5/10/50 μM) with pretreatment of 24 h (PT) against Aβ toxicity (5 μM). Results are expressed as mean SEM of four replicates (n=4). * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$ $A\beta_{1-42}$ versus Ser at different concentrations.

Discusión General

Discusión general

En esta Tesis Doctoral se ha llevado a cabo la evaluación de distintos productos fermentados elaborados a partir de puré de fresa. La fermentación de excedentes de este fruto evita pérdidas económicas, aumenta la vida útil y ofrece la posibilidad de elaborar productos innovadores para el consumidor.

Concretamente, se ha estudiado la composición antociánica, color y actividad antioxidante de fermentados alcohólicos, acéticos y glucónicos y de una bebida elaborada a partir de estos. Además se ha evaluado la bioactividad, tanto de los fermentados y del antociano mayoritario (pelargonidina 3-glucósido) así como de uno de los principales metabolitos de los antocianos (ácido protocatéquico) que a su vez está presente en fresa y en otros alimentos fermentados.

Inicialmente nos formulamos la siguiente pregunta,

¿cómo afectan los distintos tipos de fermentaciones a los compuestos antociánicos, el color y la actividad antioxidante?

Se han estudiado tres tipos de fermentaciones: alcohólica, acética y glucónica evaluándose la composición antociánica en términos de índices y la caracterización de los compuestos mediante UHPL-MS/MS Orbitrap (Q Exactive).

El Índice de Polifenoles Totales (IPF) mostró un descenso del 48 y 44 % tras la fermentación alcohólica y acética respectivamente, en comparación con el sustrato inicial.
Por otro lado la medida de los Antocianos Totales (AT), reveló un descenso de un 28 y un 72 % para ambos fermentados. La caracterización de compuestos antociánicos resultó en la identificación y cuantificación de 19, 18 y 14 antocianos en puré de fresa (sustrato de la fermentación), fermentado alcohólico y acético, respectivamente.
Se han identificado por primera vez 4 y 8 derivados antociánicos con alta precisión (masas exactas con 4 decimales y errores del orden de ppm) en los fermentados obtenidos por fermentación alcohólica y acética de fresa respectivamente (catequina-(4-8)-pelargonidina 3-glucósido, afzelequina-pelargonidina 3-glucósido, Epi-afzelequina-pelargonidina 3-glucósido, delfinidina 3-glucósido, pelargonidina 3-sambubiósido, 5-carboxipiranopelargonidina 3-glucósido, pelargonidina 3-(6-acetil)-glucósido y pelargonidina 3-(6-succinil)-arabinósido/3-(6-malonil)-ramnósido.
Tras ambas fermentaciones, los compuestos antociánicos disminuyen significativamente, siendo estas pérdidas mucho más acusadas tras la fermentación acética (91 %) en comparación con la fermentación alcohólica (20 %).

El compuesto que sufrió menores pérdidas, y por consiguiente el más estable, fue la 5-carboxipiranopelargonidina 3-glucósido. Otros autores que han evaluado el efecto de estas mismas fermentaciones en fresa, mostraron una pérdida de Antocianos Totales (AT) bastante más acusada tras la fermentación alcohólica (63-85 %) (Ubeda et al., 2013).
Asimismo, se ha descrito un 90.2 % de disminución en los AT tras la fermentación alcohólica de granada (Ordoudi et al., 2014).
Los resultados de las pérdidas de antocianos totales tras el proceso de acetificación son menores en comparación con los valores obtenidos en otros estudios (97.2-99 %) (Klopotek et al., 2005; Ubeda et al., 2013).
Por otro lado, el análisis del color de las muestras ha puesto de manifiesto que en el caso del fermentado alcohólico existe un predominio del color rojo (aumento del parámetro *a**) en comparación con el fermentado acético el cual varía hacia tonos naranjas y amarillentos (aumento del parámetro *b**).
Por tanto, se confirmó que tanto la fermentación acética como la alcohólica tienen un papel crucial en la composición antociánica y en el color de estos fermentados y que, a pesar de las pérdidas descritas, la fermentación alcohólica preserva mejor la composición antociánica que la fermentación acética o la elaboración de mermeladas (Da Silva Pinto et al., 2007).

El análisis del fermentado glucónico en el que bacterias de la especie *G. japonicus* transforman la glucosa presente en la fresa en ácido glucónico, mostró que este proceso conserva los antocianos. Este resultado puede ser explicado debido a la bajada de pH desde un valor inicial de 3.28 a un valor final 2.74 provocada por la producción de ácido glucónico obtenido tras el proceso de fermentación, lo que favorece la estabilidad de los antocianos.
Además, otros autores han demostrado que *S. cerevisiae* puede adsorber parte de los antocianos del sustrato fermentativo durante el proceso de fermentación, lo cual también puede explicar el mayor descenso de los antocianos en los fermentados alcohólicos (Morata et al., 2003).
La actividad antioxidante de los diferentes fermentados fue medida por DPPH y ORAC, técnicas *in vitro* ampliamente utilizadas para la medida de estos índices (Fernández-Pachón et al., 2004) constatándose que la actividad antioxidante no se ve afectada por la fermentación alcohólica y glucónica.
En base a estos resultados, podemos concluir que la fermentación glucónica es el proceso que mejor preserva los compuestos antociánicos, seguido de la fermentación alcohólica con un efecto moderado y la acética que destruye de manera muy acusada a los antocianos.

Por todo esto, en el capítulo 3 de la presente Tesis Doctoral, se abordó la elaboración de una bebida cuyo sustrato mayoritario fuese fermentado glucónico de fresa. La formulación de la bebida fue determinada en base a las preferencias del panel de cata del Departamento y pruebas con consumidores y consistió en: fermentado glucónico centrifugado y agua carbonatada en una proporción 80/20 (v/v) a la que se añadió un pequeña proporción de vinagre de fresa (0.05 %) que actuó como conservante y 4 gotas de Stevia© por cada 100 mL de bebida (edulcorante natural).

Una vez elaborada la bebida se planteó la siguiente cuestión

¿Cómo afectan las condiciones normales de almacenamiento a la composición antociánica, color y actividad antioxidante de esta bebida? ¿Es esta bebida estable? ¿Cuál es su tiempo de vida útil?

Para evaluar la influencia del tiempo y de la temperatura en la estabilidad de la bebida, se almacenó la misma a temperatura ambiente (25 ºC) y a temperatura de refrigeración (4 ºC) durante 0, 15, 30, 60 y 90 días periodos de tiempo en los que se muestreó.
Mediante UHPLC-MS/MS-Orbitrap (QExactive) se llegó a identificar un total de 23 compuestos antociánicos en todas las muestras, de los cuales 4 fueron por primera vez identificados con alta precisión en productos derivados de fresa (pelargonidina 3-sambubiósido, delfinidina 3-arabinósido, cianidina 3-(6-acetil)-glucósido y delfinidina 3-galactósido). Además este trabajo aportó la masa exacta con cuatro decimales de 8 compuestos no descritos anteriormente.

Comparando los datos cuantitativos del puré fresa y la bebida a tiempo 0 (bebida a 0 días), se observó una disminución importante de los compuestos antociánicos explicado por la centrifugación del fermentado glucónico y la dilución con agua al elaborar la bebida.
Sin embargo, los antocianos de tipo condensado y acilados aumentaron su concentración.
Giusti & Woltrad (2003) pusieron en evidencia que los antocianos de este tipo son compuestos que presentan mayor estabilidad y además se pueden formar en el proceso de fermentación.
De nuevo, la 5-carboxipiranopelargonidina 3-glucósido fue el compuesto más estable, no observándose cambios significativos en las muestras refrigeradas a 4 ºC en ninguno de los tiempos de almacenamiento. Los valores obtenidos tras la cuantificación de los compuestos antociánicos y el análisis del color y de la actividad antioxidante mostraron que se mantienen prácticamente inalterados a temperatura de refrigeración durante 30 días, mientras que a temperatura ambiente los antocianos comienzan a sufrir pérdidas significativas a los 15 días de almacenamiento.
Por lo tanto, el tiempo y la temperatura juegan un papel fundamental en el mantenimiento de las propiedades de esta bebida. En base a todo esto se calculó el tiempo de vida útil de la bebida resultando en: 30 días de almacenamiento a 4 ºC de temperatura.

Una vez esto nos planteamos la siguiente pregunta,

¿son estos productos potencialmente beneficiosos para la salud?

La AD es un desorden caracterizado por la formación de placas seniles en el cerebro compuestas por péptido Aβ, el cual es tóxico y lleva a la muerte neuronal. Además otro de los mecanismos moleculares de neurotoxicidad de este péptido es la producción de ROS, los cuales generan oxidación de proteínas y lípidos, daño en el ADN y son también inductores de la muerte de las neuronas (Wang et al., 2014).

En base a estos antecedentes se evaluó la capacidad de los fermentados glucónicos y alcohólicos de fresa frente a la toxicidad del péptido Aβ y a la generación de ROS mediante los ensayos *in vitro* MTT y DCFH-DA con células PC12.
Los resultados mostraron que todos los extractos antociánicos ensayados (A13I, A13F y G13I) aumentan la viabilidad celular entre un 16 y un 57 % de manera dosis dependiente, siendo este aumento mayor para el extracto glucónico que es capaz de revertir el efecto tóxico casi a nivel del control (100 % viabilidad) a una concentración de 100 µg/L.
Se han demostrado que otros extractos de frutos poseen efectos neuroprotectores similares, por ejemplo extractos de la baya de açai aumentan la viabilidad celular frente a la toxicidad del Aβ en un 25 y 29 % a 5 y 50 µg/mL (Wond et al., 2013).
Además, extractos de hojas de arándanos y de piel de uvas han demostrado un aumento de la viabilidad de entre un 15 y un 37 % respectivamente (Harvey et al., 2011).
Debido a que la pelargonidina 3-glucósido es el antociano mayoritario de la fresa se supuso que sería el compuesto que más contribuiría a este efecto, por ello se evaluó su actividad frente a la toxicidad del Aβ resultando en un incremento de viabilidad entre un 8 y un 30 % a las concentraciones ensayadas (50/100/200 µM). A 100 µM de pelargonidina 3-glucósido el aumento es de un 28 %, esta concentración corresponde a 86.4 µg/mL en el extracto, concentración a la cual la viabilidad aumenta entre 32-45 %. Este hecho indica que no solo la pelargonidina 3-glucósido es responsable de este efecto sino que han podido quedar en el extracto otros compuestos que contribuyen a la actividad protectora. Asimismo, la evaluación de la capacidad de los extractos en la atenuación de los ROS mostró que los extractos alcohólicos y glucónicos disminuyen la producción de estas especies reactivas generadas por el Aβ de manera dosis dependiente.

Así, los extractos glucónicos y alcohólicos de fresa protegen de la muerte celular y disminuyen la producción de ROS producidos por el Aβ, resultados que demuestran que los compuestos antociánicos presentan bioactividad frente a importantes procesos implicados en el desarrollo de la AD.

Teniendo en cuenta que los antocianos se metabolizan rápidamente,

¿presenta el ácido protocatéquico, uno de los principales metabolitos de antocianos, bioactividad frente a la agregación del péptido Aβ y la proteína αS y propiedades neuroprotectoras?

El ácido protocatéquico es uno de los principales metabolitos de los antocianos y se forma tras la acción de la microbiota colónica una vez han sido absorbidos del tracto gastrointestinal (Aura et al., 2005).

Se ha evaluado la bioactividad del ácido protocatéquico frente a la agregación y toxicidad del péptido Aβ y de la proteína αS (proteína responsable de la formación de los CL tóxicos para las

neuronas dopaminérgicas en la PD).
Para ello, se han utilizado diversas técnicas *in vitro*: ensayos de fluorescencia con la molécula Tioflavina T (ThT), electroforesis, western blot, Microscopía Electrónica de Transmisión (TEM) y ensayos de viabilidad celular (MTT).
Los resultados han confirmado que el ácido protocatéquico es capaz de inhibir la agregación y desestabilizar las fibras formadas de Aβ y de αS con una actividad inhibitoria y desestabilizadora de aproximadamente un 80 %. Estos valores son muy similares a la actividad que presentan otros polifenoles los cuales han demostrado actuar como potentes inhibidores a las mismas dosis ensayadas (100 µM) incluyendo: resveratrol, (+)-catequina y curcumina (Feng et al., 2009).

Los resultados obtenidos son además muy similares (63 % de inhibición a 50 µM de ácido protocatéquico) a los efectos que presenta la cianidina 3-glucósido la cual posee la capacidad de inhibir en un 60 % (50 µM) la agregación del péptido $A\beta_{25-35}$ (Tarozzi et al., 2010).
Sin embargo, la biodisponibilidad de la cianidina 3-glucósido es menor y se han encontrado menores concentraciones en fluidos biológicos en comparación con el ácido protocatéquico tras el consumo de una fuente rica en antocianos (Vitaglione et al., 2007). Asimismo, se ha demostrado que el ácido protocatéquico es capaz de atravesar la BHE ya que fue identificado en cerebro de rata tras la administración oral de un extracto de *Salvia miltiorhiza* (Zhang, et al., 2011).

De la misma forma, el ácido protocatéquico es capaz de inhibir la agregación y desestabilizar las fibras de αS con una actividad de entre un 70 y un 80 %. Ardah et al. (2014) demostraron también que el ácido protocatéquico en un ratio 1:1 (ácido protocatéquico/αS) presenta una inhibición de la agregación de un 60 %, resultado que coincide con los obtenidos en este capítulo.
En cambio, el efecto desestabilizador de fibras de αS del ácido protocatéquico no había sido descrito anteriormente.

Además este compuesto es capaz de prevenir la muerte neuronal (células PC12) frente a la toxicidad del Aβ y αS. Los resultados obtenidos demuestran un aumento del 12 % de la viabilidad a la mínima concentración de ácido protocatéquico ensayada (1 µM) frente a la toxicidad del Aβ, resultado que coincide con el obtenido por otros autores (10-15 % de aumento a 1-10 µM) (Ban et al., 2007).
Del mismo modo, la cianidina 3-glucósido aumenta la viabilidad en un 11 % a 50 µM (Tarozzi et al., 2010) en células SH-SY5Y. Esta misma protección puede alcanzarse con concentraciones mucho menores de ácido protocatéquico. Por otro lado, el ácido protocatéquico también es capaz de prevenir la muerte celular frente a la toxicidad de los oligómeros y de las fibras de αS. Nuestros resultados indican un mayor efecto neuroprotector frente a los oligómeros que frente a las fibras.

En conclusión, en este capítulo se ha demostrado que el ácido protocatéquico es un compuesto potencialmente activo en procesos implicados en la neurodegeneración provocada en la AD y en la PD. Además puede atravesar la BHE y llegar al cerebro pudiente ejercer los efectos atribuidos.

Dado que se han descrito en fresa y en alimentos fermentados otros compuestos como la melatonina y otros derivados indólicos, nos planteamos la siguiente pregunta,

¿presentan estos compuestos bioactividad frente a la agregación y la toxicidad del péptido Aβ?

La melatonina y otros compuestos indólicos han sido identificados en fresa (Badria, 2002; Stürtz et al., 2011) y en otros productos fermentados tales como vino y cerveza (Kirschbaum et al., 1999; Bartolomé et al., 2000; Manfroi et al., 2009; Wang et al., 2014).
Además de sus propiedades antioxidantes, algunos estudios han demostrado además un papel neuroprotector de la melatonina frente a diversos mecanismos moleculares implicados en la muerte neuronal en la AD (Fraser et al., 1991; Huang et al., 1997; Pappolla et al., 1998; Masilamoni et al., 2008). Sin embargo, escasos estudios han evaluado el papel de otros compuestos indólicos frente a la formación de fibras de Aβ y su protección frente a la toxicidad de este péptido.

En el capítulo 5 de la presente Tesis doctoral, se ha evaluado la bioactividad de la melatonina y de 7 intermediarios de su ruta biosintética: triptófano, triptamina, serotonina, triptofol, n-acetilserotonina, ácido 3-indolacético y etil éster de triptófano.
Para ello se han realizado los siguientes ensayos *in vitro*: ensayos de fluorescencia (ThT), electroforesis y western blot, TEM y MTT. La serotonina es el compuesto que presenta mayor actividad inhibiendo en un 80 % el proceso de fibrilogénesis y desestabilizando las fibras de Aβ de manera dosis dependiente. Estos resultados son similares a los ya demostrados por otros compuestos polifenólicos presentes en alimentos (Feng et al., 2009).

Asimismo, se evaluó el efecto neuroprotector de los compuestos obteniéndose un incremento de la viabilidad celular entre 9-25 % obteniéndose el siguiente ranking de actividad: melatonina > serotonina > triptamina > nacetil serotonina > ácido 3 indolacético > triptofol.

De este capítulo podemos concluir que la melatonina y otros compuestos indólicos, especialmente la serotonina, posee un importante efecto frente a la agregación y toxicidad del Aβ demostrado por diversas técnicas *in vitro*.

Conclusiones

Conclusiones

1. Se han identificado por primera vez 4 compuestos antociánicos con alta precisión (masa exacta con 4 decimales y errores de ppm) en fermentados alcohólicos y 8 en fermentados acéticos de fresa. La fermentación alcohólica disminuye la concentración de antocianos moderadamente (20 % de descenso). Sin embargo, el proceso de acetificación produce mayores pérdidas. La 5-carboxipiranopelargonidina fue el compuesto más estable en ambas fermentaciones. El color de los fermentados varía de rojo a naranja tras la fermentación.

2. La fermentación glucónica de fresa mantiene la composición antociánica y la actividad antioxidante siendo éstas importantes ventajas de este proceso.

3. Los extractos antociánicos de los fermentados alcohólicos y glucónicos muestran efectos neuroprotectores frente a la toxicidad inducida por el péptido Aβ en términos de viabilidad celular y ROS siendo estos efectos mayores para el fermentado glucónico.

4. Se han identificado, por primera vez y con alta precisión, los siguientes antocianos: pelargonidina 3-sambubiósido, delfinidina 3-arabinósido, cianidina 3-(6-acetil)-glucósido y delfinidina 3-galactósido en una bebida elaborada a partir de fermentado glucónico de fresa.

5. El tiempo y la temperatura son factores que influyen en la conservación de la bebida en términos del perfil antociánico y color siendo necesaria la refrigeración (4 ºC) para el mantenimiento de sus propiedades y estimándose el tiempo de vida útil en 30 días.

6. Se ha demostrado que el PCA inhibe la agregación del péptido $A\beta_{1-42}$ y de la de proteína αS y desestabiliza las fibras formadas, mediante diversas técnicas *in vitro*. Además el PCA protege de la muerte celular (PC12) producida por el $A\beta_{1-42}$ y la αS. Estos resultados demuestran que el PCA, uno de los principales metabolitos de los antocianos, presenta propiedades bioactivas frente a mecanismos que provocan la neurodegeneración en la AD y la PD.

7. Seis de los ocho compuestos indólicos evaluados (melatonina, triptamina, serotonina, N-acetilserotonina, triptofol, ácido 3-indolacético) presentan propiedades neuroprotectoras incrementando la viabilidad celular entre un 9-25 % frente a la toxicidad del péptido Aβ. Además, la serotonina inhibe el proceso de formación y desestabiliza las fibras de Aβ de manera dosis dependiente.

Conclusions

1. For the first time, 4 anthocyanin compounds have been identified with high accuracy in alcoholic fermented products and 8 anthocyanin compounds in acetic fermented products made from strawberry. Alcoholic fermentation diminishes the anthocyanin composition moderately (20 % of decrease). However, acetic fermentation process produced higher losses. Moreover, 5-carboxypyranopelargonidin 3-glucoside was the anthocyanin compound affected in a minor degree by both fermentation processes. The colour of the alcoholic and acetic fermented products made from strawberry changed during the fermentation process, varying from red to orange colour.

2. Gluconic fermentation of strawberry preserved both the anthocyanin compounds and the antioxidant activity which are main advantages of this process.

3. The anthocyanin extracts obtained from alcoholic and gluconic fermentations were protective against Aβ-peptide neurotoxicity, being the extract from gluconic fermentation the most effective, as determined by cell viability and intracellular ROS production.

4. For the first time, the following anthocyanin compounds have been identified with high accuracy in a beverage obtained from gluconic fermentation of strawberry: pelargonidin 3-sambubioside, delphinidin 3-arabinoside, cyanidin 3-(6-acetyl)-glucoside and delphinidin 3-galactoside.

5. Storage temperature and time are crucial for preserving the beverage in terms of its anthocyanin profile and colour being refrigeration (4 ºC) highly recommended to preserve the bioactive compounds and its properties for thirty days.

6. It was demonstrated that PCA can inhibit $A\beta_{1-42}$ and αS aggregation and destabilizes the pre-formed fibrils. Furthermore, PCA protects PC12 cells against Aβ and αS-induced toxicity. These effects reveal that PCA, one of the most important metabolite of anthocyanins, is a bioactive to tackle mechanisms implicated in the neurodegeneration in AD and PD.

7. Six out of eight tested compounds (melatonin, tryptamine, serotonin, N-acetylserotonin, tryptophol, 3-indolic acetic acid) present neuroprotective effects demonstrated by the increment on cell viability (between 9-25%) against Aβ toxicity. Additionally, serotonin inhibits the fibril formation process and destabilizes the pre-formed fibrils of Aβ in a dose-dependent manner.

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Anexo I

frontiers in Microbiology

MINI REVIEW
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Melatonin and Other Tryptophan Metabolites Produced by Yeasts: Implications in Cardiovascular and Neurodegenerative Diseases

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Yeast metabolism produces compounds derived from tryptophan, which are found in fermented beverages, such as wine and beer. In particular, melatonin and serotonin, may be relevant due to their bioactivity in humans. Indeed, the former is a neurohormone related to circadian rhythms, which also has a putative protective effect against degenerative diseases. Moreover, serotonin is a neurotransmitter itself, in addition to being a precursor of melatonin synthesis. This paper summarizes data reported on fermented beverages, to evaluate dietary intake. Additionally, the article reviews observed effects of yeast amino acid metabolites on the prevention of neurodegenerative diseases (Alzheimer's and Parkinson's) and angiogenesis, focusing on evidence of the molecular mechanism involved and identification of molecular targets.

Keywords: wine, beer, VEGF, β-amyloid, α-synuclein, tryptophol, serotonin

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ORIGIN, OCCURRENCE, AND DIETARY INTAKE

The presence of bioactive compounds in fermented beverages has long been observed and they have been studied with great interest. A large body of research has focused on polyphenols, in particular, since these bioactive compounds are already present in plants and released into fermented products. Yeast also transforms certain other molecules into biologically active compounds. Among these, the case of amino acid tryptophan is of interest, since it is the precursor of at least three biologically active compounds: melatonin, serotonin, and tryptophol (Mas et al., 2014).

Tryptophol is an alcohol produced by the Ehrlich pathway and it has long been detected in appreciable concentrations in wines and beers in the mg/L range (Bartolomé et al., 2000; Monagas et al., 2007). Therefore, its occurrence in beverages is widely recognized. Moreover, tryptophol has also been indicated as a quorum sensing molecule for yeast (Sprague and Winans, 2006).

Just a few years ago, melatonin was detected in wines in much lower levels: within the ng/L range. Not only was it evidenced in wines, but also in other fermented foods, as summarized in **Table 1**. Furthermore, Rodriguez-Naranjo et al. (2011) highlighted that melatonin was produced after alcoholic fermentation, pinpointing the role *Saccharomyces* plays. Indeed, different strains synthetized melatonin at different levels (Rodriguez-Naranjo et al., 2012).

The synthetic pathway of melatonin in yeast is not completely elucidated, yet it seems the formation of serotonin might be an intermediate in the pathway (Mas et al., 2014). In addition, serotonin has been detected at mg/L levels in red wine following malolactic fermentation (Wang et al., 2014). Further research is required to explore the roles of yeast and bacteria in the occurrence of these bioactive compounds in fermented products.

One of the characteristics of bioactive compounds is the minimal concentration required for them to act. The reported concentrations in wine and beer would mean that someone consuming these beverages would obtain a low daily intake of these compounds. According to WHO, the daily intake of ethanol should not exceed 30 g and 20 g for men and women, respectively. That is to say that daily intake for a man of a wine can provide up to 0.00005–0.13 mg of melatonin. In a comprehensive review summarizing the results of human intervention studies, Harpsøe et al. (2015) concluded that the bioavailability of melatonin was 15%. In our example, its bioavailable concentration should result in 1.5–4000 pg/mL of melatonin in blood. Physiological values for day plasma melatonin are very low, accounting for several pg/mL (5–10 pg/mL in human plasma) (de Almeida et al., 2011). Thus, pg/mL in plasma might be expected after dietetic intake of wine or beer, considering the values displayed in **Table 1**. Indeed, Maldonado et al. (2009) determined an increase in the concentration of plasmatic melatonin after the ingestion of a moderate dose of beer (330 mL for women volunteers, 660 mL for men). To the best of our knowledge, there is no published data on the bioavailability of serotonin after food or beverage intake.

BIOLOGICAL EFFECTS AND PREVENTION OF CHRONIC DISEASES

Literature on the biological effects of these compounds is extensive and encompasses circadian rhythm, antioxidant properties, and reproductive function. Due to the length of this mini-review, we will focus on more recent findings on the prevention of the most prevalent degenerative diseases, such as cancer, and cardiovascular and neurodegenerative diseases.

IMPLICATIONS FOR CANCER AND CARDIOVASCULAR DISEASE: THE ROLE OF ANGIOGENESIS

Angiogenesis, which consists of the formation of new blood vessels from pre-existing ones, is crucial for organ growth during embryonic development and after birth. However, in adulthood, angiogenesis plays an essential role in the pathogenesis of diverse chronic diseases, such as cancer and cardiovascular disease, involving the progression and development of the tumor, and development and destabilization of atherosclerotic plaques (Celletti et al., 2001; Bergers and Benjamin, 2003).

Angiogenesis occurs when there is an imbalance between pro-angiogenic (e.g., vascular endothelial growth factor (VEGF), basic fibroblast growth factor, alfa tumor necrotic factor, etc.) and anti-angiogenic (e.g., angiostatin and endostatin) factors. VEGF is the most active endogenous pro-angiogenic factor in humans (Giles, 2001; Dulak, 2005; Cebe-Suarez et al., 2006; Cook and Figg, 2010). It exerts its angiogenic effect by stimulating VEGF receptor 2 (VEGFR-2), which is critical for promoting the proliferation and differentiation of endothelial cells (Giles, 2001; Ferrara and Kerbel, 2005). It has been demonstrated that VEGF promotes atherosclerotic plaque progression (Celletti et al., 2001; Khurana et al., 2005) and tumor angiogenesis (Senger et al., 1993). Indeed, VEGF is a target for drug therapies that aim to

TABLE 1 | Concentration of melatonin and other tryptophan metabolites in fermented products.

Compound		Concentration		Reference
	Food			
Melatonin	Probiotic yogurt	126.7 ± 9.00	pg/g or pg/mL	Kocadağli et al. (2014)
	Kefir (fermented milk drink)	n.d	pg/g or pg/mL	
	Black olive (naturally fermented)	5.3 ± 0.10	pg/g or pg/mL	
	Bread (crumb)	341.7 ± 29.30	pg/g or pg/mL	
	Bread (crust)	138.1 ± 23.20	pg/g or pg/mL	
	Beer	94.5 ± 6.70	pg/g or pg/mL	
	Wine			
	Alaban, Sangiovese, Trebbiano (Italy)	0.6–0.4	ng/mL	Mercolini et al., 2012
	Chardonnay, Malbec, Cabernet Sauvignon (Argentina)	0.16–0.32		Stege et al., 2010
	Gropello, Merlot (Italy)	8.1–5.2		Vitalini et al., 2013
	Cabernet Saivignon, Merlot, Syrah, Tempranillo, Tintilla de Rota, Petit Verdot, Prieto Picudo, and Palomino fino (Spain)	5.1–420		Rodriguez-Naranjo et al., 2011
	Fermented orange beverage	20.0 ± 2.02	ng/mL	Fernández-Pachón et al., 2014
	Red wine	4.88–9.15	mg/L	Monagas et al., 2007
	Fermented lentils	2.70+0.25	mg/g dry material	Bartolomé et al., 2000
Tryptophol	Whole-wheat bread	–		Jiang and Peterson, 2013
	Beer	0.242 ± 0.200	mg/L	Bartolomé et al., 2000
Serotonin	Beer	3.5–24.2	mg/L	Kirschbaum et al., 1999
	Wine	2.94–5.93	mg/L	Wang et al., 2014
		1.93 ± 0.043	mg/L	Manfroi et al., 2009
		5.5	ng/mL	Mandrioli et al., 2011

inhibit VEGF signaling (Ferrara and Kerbel, 2005). Anti-VEGF antibodies, aptamers and small molecule VEGFR tyrosine kinase inhibitors have been developed and given regulatory approval for the treatment of colon, lung, breast, kidney, and liver cancer, in addition to neovascular age-related macular degeneration (Giles, 2001; Ferrara and Kerbel, 2005). However, serious side effects, such as hypertension, have been reported with prolonged use of anti-VEGF therapies (Zhu et al., 2007; Wu et al., 2008; Kappers et al., 2010). The use of natural products in reducing VEGF-induced angiogenesis may prove to be more beneficial than the current anti-VEGF drugs available (Moyle et al., 2015).

Melatonin has been associated with a decline in VEGF secretion levels in the serum of advanced cancer patients (Lissoni et al., 2001), in addition to markedly reducing the expression of VEGF in HUVEC and culture cancer cells at 1 μM and 1 mM (Dai et al., 2008; Cui et al., 2012; Álvarez-García et al., 2013; Gonçalves et al., 2014). Melatonin has also been proven to reduce endothelial cell proliferation, invasion, migration, and tube formation, through downregulation of VEGF at 1 mM (Álvarez-García et al., 2013). The possible cell signaling pathway when melatonin inhibits HUVEC proliferation has been related to the following pathway: melatonin receptors/ERK/PI3K/Akt/PKC/NF-kB (Cui et al., 2008). Additionally, Sohn et al. (2015) have recently demonstrated that melatonin (1 mM) upregulates miRNA3195 and miRNA374b, whose overexpression synergistically reduced VEGF production in hypoxic PC-3 prostate cancer cells, indicating the important role of miRNA3195 and miRNA374b in melatonin induced antiangiogenic activity. Melatonin (40 mg/kg) has also shown an antitumor effect on mammary tumor growth in mice after 21 days of treatment; the mice displayed significantly smaller tumor volume and tumor regression (Jardim-Perassi et al., 2014). Additionally, in the same study, a lower expression of VEGFR2 was observed in the melatonin-treated tumors compared to the vehicle-treated tumors. More research is consequently needed to focus on determining the molecular mechanism by which melatonin exerts its angiogenic effect and the molecular target involved.

NEURODEGENERATIVE DISEASES

Alzheimer's disease (AD) and Parkinson's disease (PD) are the most common human neurodegenerative diseases. In both cases, their incidence increases with age. The aggregation of proteins that results in different fibrillar structures is responsible for these disorders. Specifically, they are owing to the abnormal pathological assembly of amyloid- beta (Aβ), tau and α- synuclein (αS).

Indeed, several studies have demonstrated that protofibrils and oligomers of αS and Aβ are more neurotoxic than fibrils (Pike et al., 1993; Lashuel et al., 2002; Volles and Lansbury, 2003; Outeiro et al., 2008).

This review focus on the evidence of certain bioactives which can present in fermented products. However, to give a fair balance, alcohol effects on neurodegeneration have to be highlighted as it is a major component formed by yeast in alcoholic beverages. It is well-Known that alcohol intake crosses the BBB (Blood–Brain Barrier) easily producing the excessive release of neurotransmitters, oxidative stress and inflammatory response which turns out in neurotoxicity and finally cell death. (Persidsky and Potula, 2014).

ALZHEIMER'S DISEASE

Alzheimer's disease is a progressive and irreversible neurodegenerative disorder characterized by loss of memory and cognition, abstract thinking, and personality alteration. The etiology of AD is unknown in more than 90% of cases. In the pathogenesis of AD there are two principal hallmarks: neurofibrillary tangles (NFTs) and amyloid plaques. NFT are formed by the intracellular accumulation of phosphorylated tau protein and amyloid plaques, by extracellular accumulation of amyloid β peptides (Hardy and Selkoe, 2002). The amyloid beta peptide is formed via cleavage of the amyloid precursor protein (APP). In the non-amyloidogenic pathway (normal state), APP is cleaved by α-secretase, to generate sAPP (soluble N- terminal fragment), which is neuroprotective as it is involved in the enhancement of synaptogenesis, neurite outgrowth, and neuronal survival. Conversely, in the disease state, APP is cleaved by β and γ secretase, resulting in insoluble beta amyloid peptide, which has high potential for assembly and formation of toxic aggregates (Gandy, 2005).

Several mechanisms have been proposed to explain βA neurotoxicity, such as oxidative stress and loss of endogenous antioxidants (Behl et al., 1994; Abramov and Duchen, 2005; Hamel et al., 2008); mitochondrial damage, depolarization, and mitochondrial permeability transition pore opening (Moreira et al., 2001, 2010; Abramov et al., 2004, 2007); destabilization of intracellular calcium homeostasis in neurons (Bezprozvanny and Mattson, 2008); glial cells (Abramov et al., 2003, 2004), and neuroinflammation (McNaull et al., 2010).

Levels of melatonin and its precursors (serotonin and tryptophan) are significantly decreased in elderly AD individuals and are associated with the emergence of AD (Zhou et al., 2003; Greilberger et al., 2010). A growing body of evidence supports the protective role of melatonin in several molecular mechanisms implicated in the development of AD.

Among these mechanisms, the most significant one is that melatonin prevents amyloid aggregation and overproduction. This neurohormone has a great affinity for Aβ peptide, preventing amyloid fibril formation (Masilamoni et al., 2008), as determined by circular dichroism (CD) spectroscopy, electron microscopy, nuclear magnetic resonance (NMR) and electrospray ionization-mass spectrometry (ESI-MS). In particular, a hydrophobic interaction has been observed between melatonin and Aβ, specifically on the 29–40 residues of the Aβ segment (Skribanek et al., 2001). Additionally, melatonin has inhibitory effects on the formation of secondary β-sheet structures through the disruption of the histidine (His^+) and aspartate (Asp^-) salt bridges in Aβ peptide that promote fibril dissolution (Fraser et al., 1991; Huang et al., 1997; Pappolla et al., 1998).

Melatonin presents a great capacity to regulate the synthesis and maturation of APP at different levels by: decreasing its mRNA encoding β-APP (Song and Lahiri, 1997; Lahiri, 1999); blocking cAMP production, which is involved in activating the APP gene promoter, (Husson et al., 2002); and inactivating GSK-3, which promotes α-secretase mediated cleavage of APP, favoring the non-amyloidogenic pathway (McArthur et al., 1997; Zhu et al., 2001; Hoppe et al., 2010). *In vivo* studies with transgenic mice over-expressing APP (in 9–10 months they develop senile plaques) and fed with 0.5 mg/mL of melatonin in their drinking water (3 mL/day) found a reduction in important markers of the disease, including Aβ levels in the brain, and that some animals survived (Matsubara et al., 2003). The amount given to rodents are within the pharmacological dose and out of the range of the dose that can be achieved with moderate consumption of wine. Therefore it cannot be concluded that these effect will be observed in humans after wine intake. Further research is required to obtain the evidence at dietary doses.

Furthermore, melatonin exhibits a protective effect on the cholinergic system. In AD patients, a dramatic decrease of acetylcholine has been observed (Francis et al., 1999), which was related to a decrease in enzyme choline acetyltransferase (ChAT) activity and an increase in acetylcholinesterase (AChE) activity (Bieschke et al., 2005). Indeed, AChE inhibitors increase the synaptic levels of acetylcholine, which is why they are used as a treatment for mild to moderate AD. *In vivo* administration of melatonin in rats (50 mg/kg body weight) has led to significantly reduced AChE activity, with maintenance of calcium levels under conditions of oxidative stress (Masilamoni et al., 2008). Regarding ChAT, melatonin increased its activity, after 4 months of melatonin administration in rats (Feng et al., 2004).

Finally, melatonin reduces Aβ-induced oxidative stress related to reactive oxygen species (ROS) and proinflamatory cytokines, such as IL6 and IL1-β in *in vivo* studies (Masilamoni et al., 2008). As a result of these effects, melatonin protects brain neurons from damage and death by increasing viability in hippocampal neurons and glial cells following treatment with Aβ1–40, Aβ25–40, and Aβ1–28. Moreover, melatonin prevents the death of murine N2a neuroblastoma and PC12 cells by using Aβ25–35 (Pappolla et al., 1997; Ionov et al., 2011).

There is scarce literature available in relation to the activity of other tryptophan metabolites, with indole 3-acetic acid and tryptophol being the only bioactive molecules reported so far. Morshedi et al. (2007) proved the inhibitory effect of these indole derivatives on the amyloid fibrillation of hen egg-white lysozyme, which is another model for exploring the amyloidogenic mechanism.

PARKINSON'S DISEASE

Parkinson's disease is the second most common neurodegenerative disorder. Its diagnosis is based on motor abnormalities, such as resting tremor, bradykinesia, and rigidity (Duvoisin, 1992). Indeed, patients present other non-motor symptoms, such as depression, anxiety, and sleep disorders (Jenner et al., 2013). Only 10% of patients have a genetic basis, with 90% being considered sporadic cases. PD is characterized by the degeneration of the subcortical structure of the brain. Specifically, there are significant losses of dopaminergic neurons in the substantia nigra pars compacta (SNpc; Forno, 1996), although other cell populations are also susceptible to the neurodegeneration process.

α-Synuclein (αS) is a 140 amino acid and a highly abundant neuronal protein. It is found as a soluble cytoplasmatic protein associated with synaptic vesicles (Iwai et al., 1995). It is thought that it plays a role in neurotransmission and cognitive function. Although its physiological function is uncertain, the pathology is associated with the accumulation of αS aggregates, which are the main component of Lewy bodies (LBs; Spillantini et al., 1997). LBs are spherical inclusions formed by αS aggregate (99%) and other proteins.

Despite the main risk factor being aging, other possible risk factors include mutation in the SNCA (alpha-synuclein gene) and exposure to environmental toxins. The latter are also linked to metabolic abnormalities involving neurotransmitter systems (dopamine, serotonin, GABA, and glutamate), fatty acids, such as arachidonic acid-cascade, oxidative stress and mitochondrial function (Henchcliffe and Beal, 2008; Quinones and Kaddurah-Daouk, 2009; Kaidanovich-Beilin et al., 2012; Lei and Powers, 2013).

Furthermore, several studies suggest that αS oligomers and protofibrils are an important factor in neurotoxicity in PD. αS protofibrils cause membrane permeabilization, which alters cellular homeostasis and may activate an apoptotic process (Volles and Lansbury, 2003). Indeed, there is evidence to support the capacity of αS to inhibit proteosomal activity, which would prevent elimination of misfolded proteins (Giasson and Lee, 2003).

Substantial evidence also suggests that a significant factor in dopaminergic neuronal loss in the PD brain are ROS, which result from dopamine metabolism, low glutathione concentration and high levels of iron and calcium in the SNpc (Jenner and Olanow, 2006). Additionally, the brain contains high concentrations of polyunsaturated fatty acids, which, under oxidative stress, result in lipid peroxidation and generation of toxic products (Liu et al., 2008).

No treatment is currently available for the prevention or cure of PD. However, a combination of L-DOPA and antioxidants has been recommended to reduce the rate of progression of the disease, due to the decrease in dopamine levels and significant increase of oxidative stress commonly concomitant to this type of disorder (Zhu et al., 2004).

Concerning the role of melatonin in PD, several works have reported different mechanisms of action. Lin et al. (2007) demonstrated that melatonin attenuates arsenite-induced apoptosis by reducing aggregated αS levels in rat brains, by means of Western blot analysis. Additionally, Ishido (2007) showed that melatonin inhibits αS assembly, using immunostaining in rat pheochromocytoma cells.

It is also important to highlight that melatonin dose-dependently inhibits all steps of the αS assembly process. Ono et al. (2012) observed a reduction in the number of fibrils and the corresponding increase of the number of

short fibrils and amorphous aggregates (25–250 μM) using electron microscopy and thioflavin S experiments. Indeed, melatonin presents a significant destabilization effect (also dose-dependently), suggesting a decrease in beta-sheet levels. In the same study, the authors performed experiments with primary cultures of mesencephalon and neostriatum with MTT: (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) a colorimetric assay for assessing cell metabolic activity. The results showed that melatonin reduced the toxic effects of αS after pretreatment (2–6 days) with an increase in cell viability of between 56 and 97%.

In addition to this, it is well known that melatonin exhibits antioxidant properties (Reiter et al., 1997; Kotler et al., 1998). Cellular injury cause by αS-mediated perturbation of cellular redox reactions is an important mechanism proposed for PD (George et al., 2009). Melatonin has been suggested as a potential therapeutic agent in diseases where oxidative stress is thought to be a major pathogenic factor. Mayo et al. (1998) observed that this hormone was an effective free radical scavenger and that it prevented apoptosis in neuronal cells. Moreover, *in vitro* studies on MPTD-induced (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) PD in mice have shown that melatonin protects against excitotoxicity by reducing the autoxidation of dopamine. The administration of melatonin leads to normalization of complex I activity and oxidative status in mitochondria (Escames et al., 2010).

In conclusion, and based on the preceding evidence, we should consider that melatonin presents strong inhibitory effects on protofibril formation and peptide oligomerization.

AUTHOR CONTRIBUTIONS

RH-O and AC Literature search and first draft. AT, MG-P, and AM thorough revision and discussion and final document.

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Anexo II

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Effects of gluconic and alcoholic fermentation on anthocyanin composition and antioxidant activity of beverages made from strawberry

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ABSTRACT

Strawberry is a very perishable fruit, well-known for being a source of bioactive compounds. The elaboration of the beverages by alcoholic and gluconic fermentation process has been explored as a worthy strategy for preventing food losses as well as preserving bioactive compounds with antioxidant properties.

To this end, this paper aims to characterize the anthocyanin composition of the resulting beverages and to evaluate their antioxidant properties with *in vitro* assays (ORAC, DPPH). Additionally, the protective effect against amyloid-β (Aβ) peptide toxicity in terms of Reactive Oxygen Species (ROS) production and PC12 cells viability was determined.

Eleven anthocyanin compounds were identified and quantified by UHPLC-DAD-MS. Pelargonidin 3-glucoside and its derivatives were the major compounds. Gluconic fermentation preserved anthocyanin composition being an advantage of this innovative process. Accordingly the values of antioxidant activity were higher for gluconic than alcoholic fermented beverages. Indeed, both of them increased cell viability (16–57% $p < 0.05$) and attenuate the oxidative stress triggered by Aβ (13–38% $p < 0.05$).

1. Introduction

Strawberry is an important fruit crop worldwide, especially for fresh consumption. An alternative for avoiding economic loss due to its perishable nature is the elaboration of derivatives products such as jams, yoghourts, products for biscuits or cakes and beverages made from strawberry pureés.

Recently, different studies summarized the evidence for the health benefits of strawberry and other berry fruits (Basu, Nguyen, Betts, & Lyons, 2014; Giampieri et al., 2015). Indeed, strawberry is a good source of bioactive compounds. Furthermore, the antioxidant properties of strawberry have been attributed to its polyphenol and vitamin content, being ascorbic acid, ellagitanins and anthocyanins the greatest contributors to its antioxidant capacity (Aaby, Ekeberg, & Skrede, 2007; Manganaris, Goulas, Vicente, & Terry, 2014). Recently, strawberries were included among the 100 richest sources of dietary polyphenols and also listed in rankings of 89 foods and beverages that provide more than 1 mg of polyphenols per serving (Pérez-Jiménez, Neveu, Vos, & Scalbert, 2010). Anthocyanins are responsible for the red color of berry fruits, such as

Abbreviations: Aβ, amyloid-β; AAPH, 2,2′-diazo-bis-amidinepropane-dihydrochloride; DCFDA, 2′,7′-dichlorofluorescin diacetate; DPPH, 2,2-diphenyl-1-picrylhydrazyl; IC_{50}, Half Maximal Inhibitory Concentration; MTT, Thiazolyl Blue Tetrazolium Bromide; ORAC, Oxygen Radical Absorbance Capacity; ROS, Reactive Oxygen Species; UHPLC, Ultra High-Performance Liquid Chromatography.

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blueberries, blackberries and strawberries. It is well known that pelargonidin 3-glucoside is the major anthocyanin in strawberry (150–650 mg kg^{-1} of fresh weight) (García-Viguera, Zafrilla, & Tomás-Barberán, 1998; Lopes-da-Silva, Escribano-Bailón, Pérez Alonso, Rivas-Gonzalo, & Santos-Buelga, 2007). Indeed, there are crucial factors that influence significantly the stability of anthocyanin compounds such as process, time, and storage temperature (Clifford, 2000). Several efforts have been done to diminish the effect of process in the composition of products made from strawberry using different production systems and the employment of modified atmosphere in the storage (Fan et al., 2012; Oliveira et al., 2015).

Furthermore, the production of strawberry drinks is an innovative trend. In particular, the fermentation by *Gluconobacter japonicus* which transforms the glucose content of the fruit into gluconic acid to keep the fructose as sweetener (Cañete-Rodríguez et al., 2015). Additionally, alcoholic fermentation by *Saccharomyces cerevisiae*, is used to elaborate strawberry beverages (Hidalgo, Torija, Mas, & Mateo, 2013) The impact of gluconic fermentation has been evaluated in terms of amino acids and biogenic amines (Ordóñez et al., 2015). Besides, non-anthocyanin composition of these beverages (gluconic and alcoholic) has been described before showing their potential as a source of bioactive compounds (Álvarez-Fernández, Hornedo-Ortega, Cerezo, Troncoso, & García-Parrilla, 2014; Álvarez-Fernández, Cerezo, Cañete-Rodriguez, Troncoso, & García-Parrilla, 2015).

Among the healthy properties of strawberries, neuroprotective effects due to the anthocyanin content have been reported (Giampieri et al., 2015). Hence, recent studies show the effectiveness of anthocyanins against Aβ toxicity. Indeed, Badshah, Kim, and Kim (2015) demonstrated that an anthocyanin extract (cyanidin 3-glucoside, delphinidin 3-glucoside and petunidin 3-glucoside) of black soybean decreased the neuronal death in HT22 cells. Additionally, cyanidin 3-glucoside can inhibit $A\beta_{25-35}$ spontaneous aggregation into oligomers and their neurotoxicity in human neuronal SH-SY5Y cells (Tarozzi et al., 2010). To the best of our knowledge, the protective effect against Aβ peptide has not been explored neither with strawberry or its derivatives nor for pelargonidin and derivatives compounds. Therefore, our work intends to explore the hypothetical activity these compound and beverages may present.

The aims of this paper are to (i) characterize the anthocyanin composition of fermented beverages elaborated from strawberry, (ii) to estimate the effect of alcoholic and gluconic fermentation on anthocyanin compounds, (ii) to evaluate their bioactive potential.

2. Material and methods

2.1. Samples

Hudisa Desarrollo Industrial S.A. (Lepe, Spain) provided strawberry purée. The process of elaboration of the strawberry mash is summarized as follows: the fruit is received, selected, cleaned and the stems and leaves are eliminated. After the strawberry has been mashed, an inactivation enzymatic (2 min, 55 ºC–65 °C) is performed, followed by a pasteurization process (3 min, >90 °C). Finally, the temperature is reduced to 5 °C. The mash is sieved to remove the seeds.

In this study, two harvests were analyzed (2012 and 2013). These purées were frozen (−20 °C) until fermentation was carried out. Alcoholic and gluconic fermentations were conducted in the Department of Inorganic Chemistry and Chemical Engineering of the University of Córdoba (Córdoba, Spain). Fermentation conditions were previously described (Álvarez-Fernández et al., 2014, 2015). Alcoholic fermentation was carried out with a *S. cerevisiae* (CET 13057 isolated from native strawberry yeast) used as a starter for the submerged fermentation process. The fermentation process was as follows: 3.6 L of strawberry purée were placed into the bioreactor and the conditions set (pH 3.32, 29 °C, 26.20 rad s^{-1}); the medium was saturated with oxygen only at the beginning of the fermentation process, before adding the inoculum (10% (w/v) glucose, 0.1% (w/v) $MgSO_4$, 0.2% (w/v) KH_2PO_4, 0.3% (w/v) $(NH_4)_2SO_4$, 0.4% (w/v) yeast extract and 0.36% (w/v) bacteriological peptone). The end of the fermentation process was established when the glucose had been totally consumed and final pH was 3.30.

For gluconic fermentation, 3 L of strawberry purée substrate were placed into the bioreactor and the conditions set (pH 3.24, 29 °C, 20% O_2 and 1250 g); after 10–20 min, 125 ml of inoculum of *G. japonicus* strain E1 were added (5% (w/v) glucose, 1% (w/v) bacteria extract and 2% (w/v) bacteriological peptone) and mixed for 20–30 min, then the initial sample was taken. The end of the fermentation process was established when the glucose had been totally consumed and final pH was 2.74.

Eight alcoholic fermentation (code A) experiments were performed: four with purées from the 2012 harvest (code 12) and four with those from the 2013 harvest (code 13). Additionally, six gluconic fermentation experiments (code G) were performed: four with purées from the 2012 harvest and two from the 2013 harvest. Samples were taken at the initial point of the fermentation experiment (I), the final point (F) and after pasteurization of the fermented product (FP). Pasteurization was carried out at 70–80 °C for 15 min. All samples were frozen until analysis. Table 1 displays the codes of the samples used in this study.

2.2. Chemicals and reagents

Amberlite XAD7HP, Dimethyl sulfoxide (DMSO), Dulbecco's modified Eagle's medium (DMEM)-Glutamax, Trypsine-EDTA, Thiazolyl Blue Tetrazolium Bromide (MTT), Phosphate Buffered Saline (PBS), L-glutamine, fetal horse serum and fetal bovine serum, streptomycin, 2′,7′-dichlorofluorescin diacetate (DCFH-DA), 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2′-diazo-bis-amidinepropane-dihydrochloride (AAPH), and Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic) were purchased from Sigma (Steinheim, Germany). Fluorescein sodium was obtained through Fluka (Steinheim, Germany).

VWR Chemicals (Llinars del Vallés, Barcelona) supplied methanol and acetic acid. Formic acid and acetonitrile were obtained by Fisher Chemical. Pelargonidin 3-glucoside was obtained from Chloride (Cromadex Inc., USA). Cells PC12-Adh were supplied by ATCC® CRL-1721.1™ (Manassas, USA) and Amyloid β-protein 25–35 ($A\beta_{25-35}$) by Synvec (Bordeaux, France).

Table 1
Sample codes.

Codes	Sample
A 12 I	Alcoholic, 2012 Harvest, Initial
A 12 F	Alcoholic, 2012 Harvest, Final
A 12 FP	Alcoholic, 2012 Harvest, Pasteurized
A 13I	Alcoholic, 2013 Harvest, Initial
A 13 F	Alcoholic, 2013 Harvest, Final
A 13 FP	Alcoholic, 2013 Harvest, Pasteurized
G 12 I	Gluconic, 2012 Harvest, Initial
G 12 F	Gluconic, 2012 Harvest, Final
G 12 FP	Gluconic, 2012 Harvest, Pasteurized
G 13 I	Gluconic, 2013 Harvest, Initial
G 13 F	Gluconic, 2013 Harvest, Final
G 13 FP	Gluconic, 2013 Harvest, Pasteurized

2.3. Sample preparation

An Amberlite XAD7HP column (30 × 1.5 cm) was conditioned with 200 mL of methanol and then 200 mL of water. A total of 20 g of sample (gluconic and alcoholic fermented samples) were diluted with water (1:1, w/v). The column was loaded with the diluted sample and cleaned with water to eliminate sugars, acids and polar compounds. Subsequently, the anthocyanin fraction was eluted with a mixture of methanol: acetic acid (19:1); flow rate 1 drop s^{-1}. This fraction was collected and concentrated with a rotary evaporator under vacuum (Büchi Rotavapor, R-200/205), frozen (−80 °C) and finally freeze-dried. A total of approximately 20 mg of this extract was obtained from 20 g of sample. The respective extracts were used for the subsequent analysis.

2.4. Analysis of anthocyanin compounds

Analyses were carried out using a UHPLC system model 1290 Infinity (Agilent, Palo Alto, CA), equipped with a binary pump (Agilent Technologies, 1290 VL G4220B), an autosampler (Agilent Technologies, 1290 sampler, G4226A) and a DAD detector (Agilent Technologies, G1316C). The UHPLC system was coupled to a Bruker mass spectrometer (model Esquire 3000+) with electrospray ionization (ESI), Atmospheric Pressure Chemical Ionization (APCI) and an Ion Trap analyzer. Samples were filtered through a Millipore PTFE 0.45 μm filter before injection.

The sample volume injected was 1 μL. A SB-C18 column (2.1 × 100 mm, 1.8 μm) (Agilent, USA) was used. Two different solvents were used as a mobile phase: solvent A (water/formic acid 90:10 v/v) and solvent B (acetonitrile/formic acid 90:10 v/v), at a flow rate of 0.4 ml min^{-1} and a gradient as follows: 0 min 1% B, 9 min 20% B, 13.44 min 100% B, 14.55 100% B and 14.77 1% B.

Data were processed using HyStar 3.2 software (Bruker). The MS/MS parameters were as follows: positive mode; capillary tension: −3700 V; nebulizer: 40psi; dry gas: 10 l min^{-1}; dry temperature: 365 °C; and scan range, *m/z*: 100 to 1200.

Anthocyanins in strawberry were quantified using the areas of their chromatographic peaks at 520 nm by comparison with a standard pelargonidin 3-glucoside calibration curve. Strawberry extracts were analyzed in duplicate.

2.5. Antioxidant activity

2.5.1. DPPH method

The DPPH free radical scavenging activity assay was performed according to Katalinic, Milos, Kulisic, and Jukic (2006) with slight modifications and using 96-well plates. A total of 50 μL of extracts (5–100 μg mL^{-1}) were added to each well and completed to a final volume of 200 μL with 150 μM DPPH methanolic solution. Plates were shaken for 10 s and then absorbance was measured in a multi-detector microplate reader at 517 nm (Synergy HT, Biotek®). The inhibition percentage of DPPH radical (I%) was calculated as follows:

$$I\% = ((Abs_{DPPH} - Abs_S) / Abs_{DPPH}) \times 100$$

Where Abs_{DPPH} is the absorbance of the control without sample and Abs_S is the absorbance of the tested sample measured at 20 min.

Sample concentration providing 50% inhibition (IC_{50}) was calculated from the graph, plotting inhibition percentage against sample concentration (μg mL^{-1}). All experiments were performed in triplicate.

2.5.2. ORAC test

The procedure is based on a previously reported method (Ou, Hampsch-Woodill, & Prior, 2001) with slight modifications: 50 μL of sample or Trolox are mixed with 100 μL of fluorescein (45 mM) and 50 μL of AAPH (15 mM). Fluorescence was recorded for 90 min (excitation wavelength was set at 485 nm; emission wavelength at 528 nm). Measurements were taken in triplicate in a multi-detector microplate reader (Synergy HT, Biotek®). Trolox was used as a calibration standard at the 0.5–9.5 μM concentration range established previously (Cerezo, Cuevas, Winterhalter, García Parrilla, & Troncoso, 2010).

Fluorescein fluorescence was recorded every 5 min after the addition of AAPH, until the fluorescence was <5% of the initial reading. The final results were calculated using the areas under the fluorescein decay curves between the blank and the sample and were expressed as μmols of Trolox g^{-1} of fresh weight.

2.6. Cell culture

PC12 cells derived from rat pheochromocytoma were cultured in a DMEM-Glutamax medium containing 100 units mL^{-1} penicillin, 100 μg mL^{-1} streptomycin, 15% fetal horse serum and 2.5% fetal bovine serum and maintained at 37 °C in a humidified incubator with 5% CO_2.

PC12 were subcultured at a density of 30 000 cells per well, in 96-well culture plates in 200 μL of culture medium. After 24 h, cells were incubated with strawberry extracts (10–100 μg mL^{-1}) or pelargonidin 3-glucoside (50–300 μM), in the presence or absence of $A\beta_{25\text{-}35}$ (10 μM and 20 μM for viability and ROS measurement, respectively), in a serum-free culture medium. Pelargonidin 3-glucoside was dissolved in DMSO at a final concentration of 0.1% (v/v) and extracts were dissolved in water (1 mg mL^{-1}).

2.7. MTT reduction cell viability

After treatment (24 h), PC12 cells were incubated with 0.5 mg mL^{-1} of MTT for 3 h at 37 °C. The resulting crystals that formed were dissolved with 100 μL of DMSO and were finally determined by measuring absorbance, using a microplate reader MRXII (Dynex), at a wavelength of 540 nm. Data are expressed as means ± SEM.

2.8. Intracellular ROS measurement

Generation of ROS in cells was analyzed using a fluorometric probe (DCFH-DA). After treatment (6 h), cells were washed with PBS, and 5 μM of DCFH-DA was added. The fluorescence intensity was immediately quantified for 30 min at 37 °C using a spectrofluorometer (FLUOstar Optima, BMG Labtech). The wavelengths of excitation and emission used to detect the ROS were 485 nm and 520 nm, respectively. All experiments were performed in the dark. Results were given as the percentage of ROS production compared with the untreated control cells.

2.9. Statistical analysis

One-way analysis of variance (ANOVA test) ($p < 0.05$) was used to explore significant differences in anthocyanin composition before and after the fermentation and pasteurization processes, using statistical software (Statsoft, 2004). The IC_{50} was calculated using Graphpad Prism 6 software. For ROS and cell viability measurements, the statistical tests were performed with one-way ANOVA (statistical software), followed by Dunnett's multiple comparison post-hoc test. Significance was set at $p < 0.05$.

3. Results and discussion

3.1. Anthocyanin characterization

The chromatogram profile of the strawberry samples is presented in Fig. 1. A total of eleven compounds were identified in both alcoholic and gluconic fermented products by LC-DAD-MS/MS. Table 2 summarizes the retention time and mass spectrometry characteristics of those compounds compared with data reported in the literature.

Pelargonidin 3-glucoside (peak 7) was the major compound, followed by pelargonidin 3-rutinoside (peak 8) and cyanidin 3-glucoside (peak 5), in accordance with previously reported data (García-Viguera et al., 1998; Pérez-Jímenez et al., 2010).

Catechin-(4–8)-pelargonidin 3-glucoside and epicatechin-(4–8)-pelargonidin 3-glucoside (peaks 1 and 2, respectively) were also identified. They both presented identical molecular ions at *m/z* 721 and fragmentation patterns (559/407/313), but had different retention times. Additionally, other anthocyanin linked flavonols, such as (epi) afzelechin-pelargodinin 3-glucoside (peak 4) were

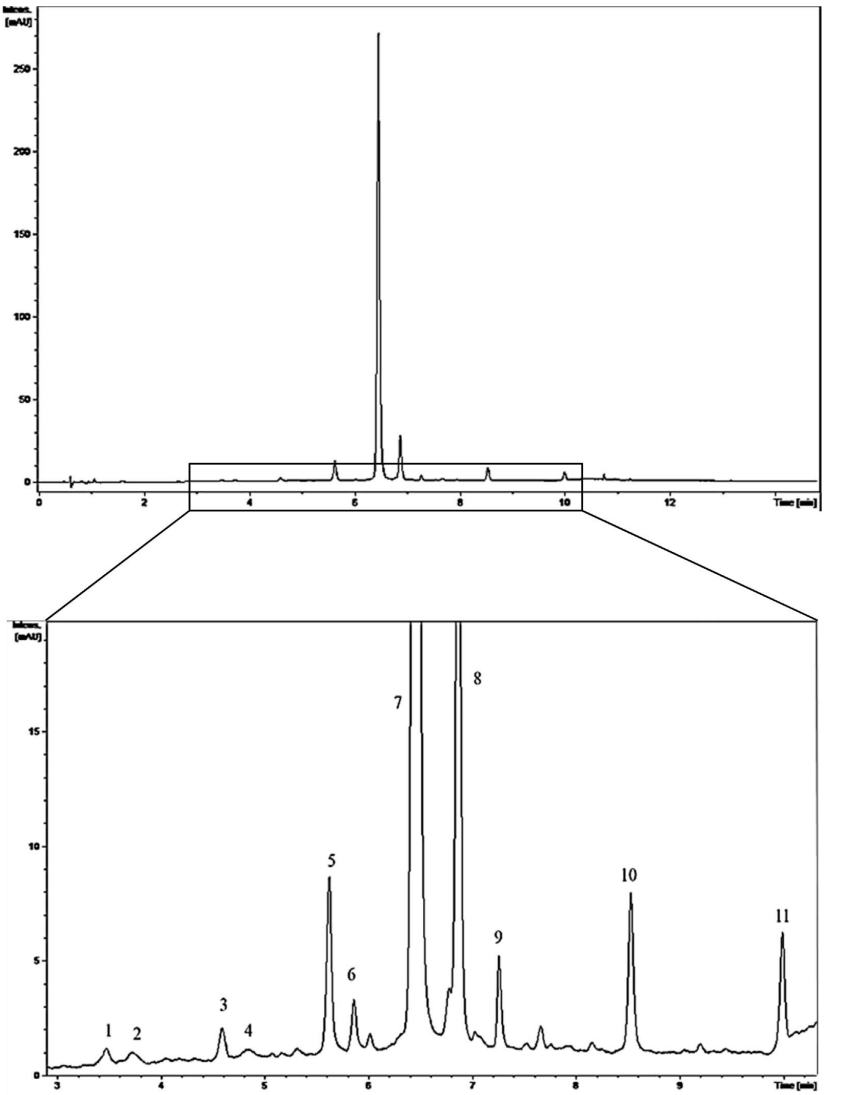

Fig. 1. Chromatogram and chromatogram (zoom) recorded at 520 nm showing the anthocyanin profile of the strawberry samples. 1: Catechin-(4–8)-pelargonidin 3-glucoside; 2: Epicatechin-(4–8)-Pelargonidin 3-glucoside; 3: Delphinidin 3-glucoside; 4: (epi)Afzelechin-pelargodinin 3-glucoside; 5: Cyanidin 3-glucoside; 6: Pelargonidin 3,5-diglucoside; 7: Pelargonidin 3-glucoside; 8: Pelargonidin 3-rutinoside; 9: 5-carboxypyranopelargonidin 3-glucoside; 10: Pelargonidin 3-(6″-malonylglucoside); 11: Pelargonidin 3-acetylglucoside.

Table 2
Identification of anthocyanin compounds of alcoholic- and gluconic-fermented products of strawberry purée.

Peak	T_R[a] (min)	Molecular ion [M^+] (*m/z*)	MS^2 (*m/z*)	Identification	References
1	3.5	721	559/407/313	Catechin-(4,8)-pelargonidin 3-glucoside	Lopes-da-Silva et al., 2007; Fossen, Rayyan, & Andersen, 2004
2	3.7	721	559/407/313	Epicatechin-(4,8)-pelargonidin 3-glucoside	Lopes-da-Silva et al., 2007
3	4.4	465	303	Delphinidin 3-glucoside	Cerezo et al., 2010
4	4.6	705	545/407/313	(epi)Afzelechin-pelargonidin 3-glucoside	Lopes-da-Silva et al., 2007
5	5.6	449	287	Cyanidin 3-glucoside	Wu & Prior, 2005; Lopes-da-Silva et al., 2002
6	6.0	595	433/271	Pelargonidin 3,5-diglucoside	Lopes-da-Silva et al., 2007; Cerezo et al., 2010; Wu & Prior, 2005
7	6.5	433	271	Pelargonidin 3-glucoside	Wu & Prior, 2005; Lopes-da-Silva et al., 2002
8	6.8	579	433/271	Pelargonidin 3-rutinoside	Wu & Prior, 2005; Lopes-da-Silva et al., 2002
9	7.3	501	339	5-carboxypyranopelargonidin 3-glucoside	Cerezo et al., 2010
10	8.6	519	271	Pelargonidin 3-(6″-malonylglucoside)	Wu & Prior, 2005
11	9.9	475	271	Pelargonidin 3-acetylglucoside	Lopes-da-Silva et al., 2007; Wu & Prior, 2005

[a] T_R, retention time.

identified. We also detected delphinidin 3-glucoside (peak 3) and 5-carboxypyranopelargonidin 3-glucoside (peak 9) in these samples, which were previously reported for the first time by our group in *Camarosa* strawberry purée (Cerezo et al., 2010). Furthermore, other pelargonidin derived compounds were identified as follows: peak 10 showed a molecular ion at *m/z* 519 and a fragmentation pattern at *m/z* 271 corresponding to pelargonidin 3-(6″-malonylglucoside); peak 11 was consistent with pelargonidin 3-acetylglucoside, showing a molecular ion at *m/z* 475 and fragmentation pattern at *m/z* 271; and peak 6 corresponded to pelargonidin 3,5-diglucoside (*m/z* 595/433/271), all of them in accordance with previous reports (Lopes-da-Silva, de Pascual-Teresa, Rivas-Gonzalo, & Santos-Buelga, 2002, 2007).

3.2. Effects of alcoholic and gluconic fermentation processes

Table 3 displays anthocyanin composition data after alcoholic fermentation. Two of the major compounds cyanidin 3-glucoside and pelargonin 3-glucoside decreased significantly ($p < 0.05$) after the fermentation process (51.5–38.3% and 56.3–38.7% respectively, considering both harvests). Other authors have also reported a reduction in total anthocyanins (85–63%) in strawberry wine as a result of the alcoholic fermentation process (Klopoyek, Otto, & Böhm, 2005; Ubeda et al., 2013). Our results reveal that the total anthocyanin decrease is due specifically to cyanidin 3-glucoside and pelargonidin 3-glucoside. In contrast, pelargonidin 3,5-diglucoside significantly ($p < 0.05$) increased its concentration after the fermentation process, in both harvests (2.5 and 6.2 times higher, respectively).

On the other hand, gluconic fermentation preserved all the anthocyanin compounds, since no significant changes took place after this process (Table 4). Different factors could explain the observed results. Firstly, the decrease in the pH value obtained after gluconic fermentation (from an initial 3.24 to 2.74) while alcoholic fermentation does not change significatively (from 3.32 to 3.30). The acidic pH obtained after the conversion of glucose into gluconic acid by *G. japonicus* probably stabilizes these compounds. On the other hand, an adsorption mechanism between the yeast (*S. cerevisiae*) and anthocyanin compounds has been stated as responsible of the decrease on anthocyanin in alcoholic fermented final products (Morata et al., 2003). Our work reveals that *G. japonicus* does not interact with anthocyanin compounds in such an extent that could make a significant difference in the beverage.

Gluconic fermentation is therefore an advantageous process for maintaining anthocyanin compounds, as has also been shown for non-anthocyanin phenolic compounds in the gluconic fermentation of strawberry (Álvarez-Fernández et al., 2014).

3.3. Antioxidant activity, DPPH and ORAC method

Table 5 shows antioxidant activity expressed in Half Maximal Inhibitory Concentration (IC_{50}) (DPPH method) and μmols of Trolox g^{-1} of fresh weight (ORAC method). The results of the ORAC assays showed similar values in all cases (I, F and P). Antioxidant activity is conserved in all extracts, regardless the process involved. Furthermore, DPPH assays show the same tendency and it can be concluded that gluconic and alcoholic fermentation leave *in vitro* antioxidant activity practically unchanged.

3.4. Cell viability of PC12 and ROS production

To evaluate the protective effects of strawberry fermented extracts, their capacity to protect rat pheochromocytoma derived PC12 cells from Aβ peptide induced toxicity was measured. An MTT assay was used to determine cell viability. Three extracts (I, and A and G final samples, 2013 harvest) were selected in order to identify the impact of alcoholic and gluconic fermentation on the activity of the extract. In particular, those extracts with higher concentration

Table 3
Concentration (mg kg^{-1}) of alcoholic-fermented extracts.

Compounds	A12I	A12F	A12FP	A13I	A13F	A13FP
Delphinidin 3-glucoside	0.64 ± 0.15^{ab}	0.30 ± 0.10^{a}	0.36 ± 0.16^{b}	0.69 ± 0.13^{b}	0.52 ± 0.22	0.41 ± 0.15^{b}
Cyanidin 3-glucoside	3.30 ± 0.80^{ab}	1.60 ± 0.40^{a}	1.90 ± 0.40^{b}	4.70 ± 0.60^{ab}	2.90 ± 0.50^{a}	2.70 ± 0.30^{b}
Pelargonidin 3,5-diglucoside	0.10 ± 0.07^{ab}	0.25 ± 0.10^{a}	0.21 ± 0.11^{b}	0.13 ± 0.04^{ab}	0.81 ± 0.13^{ac}	0.65 ± 0.04^{bc}
Pelargonidin 3-glucoside	61.20 ± 13.08^{ab}	26.7 ± 7.8^{ac}	34.90 ± 5.40^{bc}	116.2 ± 13.3^{ab}	71.72 ± 10.24^{a}	66.07 ± 8.13^{b}
Pelargonidin 3-rutinoside	10.35 ± 2.02^{ab}	7.80 ± 1.80^{a}	8.11 ± 1.14^{b}	11.90 ± 1.60^{b}	11.90 ± 1.80^{c}	9.90 ± 1.60^{bc}
5-carboxypyranopelargonidin 3-glucoside	0.73 ± 0.11	0.67 ± 0.09	0.68 ± 0.15	0.97 ± 0.12	1.06 ± 0.16	0.94 ± 0.18
Pelargonidin 3-(6″-malonylglucoside)	1.31 ± 0.22^{ab}	0.88 ± 0.20^{a}	0.99 ± 0.14^{b}	3.00 ± 0.40^{b}	2.80 ± 0.40^{c}	2.46 ± 0.23^{bc}
Pelargonidin 3-acetylglucoside	0.65 ± 0.15^{a}	0.48 ± 0.13^{a}	0.52 ± 0.10	1.80 ± 0.30^{b}	1.72 ± 0.23	1.50 ± 0.24^{b}

[a] Superscript letter indicates significant difference ($p < 0.05$) between Initial (I) and Final point (F) and Final Pasteurized point (FP) within the same harvest.
[b] Superscript letter indicates significant difference ($p < 0.05$) between Initial (I) and Final point Pasteurized (F) and Final Pasteurized point (FP) within the same harvest.
[c] Superscript letter indicates significant difference ($p < 0.05$) between Final point (F) and Final point Pasteurized (F) and Final Pasteurized point (FP) within the same harvest.
Sample identification in Table 1.

Table 4
Concentration (mg kg^{-1}) of gluconic-fermented extracts.

Compounds	G12I	G12F	G12FP	G13I	G13F	G12FP
Delphinidin 3-glucoside	0.46 ± 0.10	0.35 ± 0.20	0.33 ± 0.22	0.41 ± 0.04[b]	0.60 ± 0.40	0.85 ± 0.03[b]
Cyanidin 3-glucoside	2.80 ± 0.30[b]	2.30 ± 0.70	2.10 ± 0.50[b]	4.79 ± 0.54	5.20 ± 0.50	5.26 ± 0.22
Pelargonidin 3,5-diglucoside	0.09 ± 0.03	0.10 ± 0.05	0.06 ± 0.01	0.12 ± 0.01	0.14 ± 0.03	0.15 ± 0.00
Pelargonidin 3-glucoside	51.77 ± 6.06[b]	44.78 ± 12.24	41.60 ± 9.80[b]	122.19 ± 11.22	129.1 ± 11.5	131.91 ± 5.18
Pelargonidin 3-rutinoside	8.07 ± 1.24	7.30 ± 2.30	6.60 ± 1.70	12.20 ± 0.40[a]	13.60 ± 0.30[a]	13.4 ± 0.9
5-carboxypyranopelargonidin 3-glucoside	0.57 ± 0.12[b]	0.61 ± 0.12	0.68 ± 0.08[b]	0.50 ± 0.03[ab]	1.12 ± 0.15[a]	1.15 ± 0.12[b]
Pelargonidin 3-(6″-malonylglucoside)	0.99 ± 0.15	0.90 ± 0.16	0.91 ± 0.04	3.60 ± 0.40	3.26 ± 0.20	3.25 ± 0.17
Pelargonidin 3-acetylglucoside	0.53 ± 0.09	0.48 ± 0.14	0.46 ± 0.12	1.86 ± 0.22	1.95 ± 0.12	1.97 ± 0.05

[a] Superscript letter indicates significant difference ($p < 0.05$) between Initial (I) and Final point (F) and Final Pasteurized point (FP) within the same harvest.
[b] Superscript letter indicates significant difference ($p < 0.05$) between Initial (I) and Final point Pasteurized (F) and Final Pasteurized point (FP) within the same harvest.
[c] Superscript letter indicates significant difference ($p < 0.05$) between Final point (F) and Final point Pasteurized (F) and Final Pasteurized point (FP) within the same harvest.
Sample identification in Table 1.

Table 5
Antioxidant activity for DPPH (IC_{50}: Half Maximal Inhibitory Concentration) and ORAC (μmols Trolox g^{-1} fresh weight) methods.

	DPPH		ORAC
Samples	IC_{50} (μg mL^{-1})	CI (confidence interval)	(μmols Trolox g^{-1} fresh weight)
A12I	16.32	14.81–17.97	10.4 ± 2.3
A12F	17.57	16.30–19.93	12.8 ± 2.4
A12FP	19.99	17.06–23.42	8.5 ± 1.8
A13I	15.88	14.16–17.82	19.1 ± 1.0
A13F	15.55	14.17–17.06	14.8 ± 1.2
A13FP	18.20	15.39–21–53	15.1 ± 0.8
Samples	IC_{50}	CI (Confidence Interval)	(μmols Trolox g^{-1} fresh weight)
G12I	17.93	15.36–20.94	9.4 ± 1.7
G12F	20.18	18.86–21.59	12.2 ± 1.1
G12FP	19.31	15.46–24.11	7.6 ± 2.9
G13I	18.26	13.90–24.00	20.7 ± 2.7
G13F	14.86	13.19–16.74	13.9 ± 0.8
G13FP	17.04	14.07–20.65	12.1 ± 1.5

Sample identification in Table 1.

in cyanidin 3-glucoside, pelargonidin 3-glucoside, pelargonidin 3-rutinoside and pelargonidin 3-(6″-malonylglucoside) according to the values displayed in Tables 3 and 4 were used for further experiments.

First, the cytotoxic potential of the extracts at different concentrations ranging from 10 to 200 μg mL^{-1} on PC12 cells was measured. We observed a toxic effect for PC12 cells at 200 μg mL^{-1} in all tested extracts (data not given). Fig. 2 displays cell viability expressed as a percentage relative to the untreated control cells. After exposure to $A\beta_{25\text{-}35}$ alone, viability decreased by more than 60%, compared with the control. However, when cells were treated with $A\beta_{25\text{-}35}$ in the presence of the strawberry extracts, cell viability increased significantly ($p < 0.05$) (16–57%), in a dose-dependent manner. Specifically, 100 μg mL^{-1} of gluconic extract reversed amyloid-induced toxicity and increased cell viability up to near the control level (Fig. 2). Other authors have reported that acai berry extracts (50 μg mL^{-1}) did not provide any significant degree of protection against $A\beta_{25\text{-}35}$-mediated loss of cell viability PC12; however, they had an effect when tested with $A\beta_{1\text{-}42}$ at 5 μM, with 25 and 29% increased cell viability at 5 and 50 μg mL^{-1}, respectively (Wond, Musgrave, Harvey, & Smid, 2013). Additionally, blueberry leaf extracts (50–100 μg mL^{-1}) caused a small cell viability increase of 6–15% on $A\beta_{25\text{-}35}$-induced cytotoxicity in PC12 cells (Jeong et al., 2013). Harvey, Musgrave, Ohlsson, Franson, and Smid (2011) found that grape-skin extract tested at different concentrations of $A\beta_{1\text{-}42}$ provided protection against amyloid toxicity (37%) in PC12 cells. As far as we know, the strawberry extract studied in this paper shows one of the highest protective effects against Aβ-induced damage.

The effect of the extracts on cell viability could be explained as being due to the presence of pelargonidin 3-glucoside, which is the major compound in our extracts. For this reason, we evaluated the cytoprotective ability of pelargonidin 3-glucoside standard on PC12 cells. Cell viability was decreased after cells were exposed to 300 μM (data not shown). However, a protective effect was determined at concentrations of 50, 100 and 200 μM (21.6, 43.2 and 86.4 μg mL^{-1}, respectively), in a dose-dependent manner (Fig. 2B). In gluconic fermented extract (G13F) at the highest concentration tested (100 μg mL^{-1}), pelargonidin 3-glucoside concentration is 12.9 μg mL^{-1}. Since the pelargonidin 3-glucoside at concentration 21.6 μg mL^{-1} (= 50 μM) increased cell viability by just 10%, the protective effects of gluconic fermented extract are not only due to the pelargonidin 3-glucoside. Other anthocyanins described here and polyphenols previously identified in strawberry may contribute to the protective activity in our extracts (Cerezo et al., 2010). Indeed, other authors have reported that cyanidin 3-glucoside and ellagic acid also contribute to the neuroprotective effect against $A\beta_{25\text{-}35}$ and $A\beta_{1\text{-}42}$, respectively, in SH-SY5Y cells (Feng et al., 2009; Tarozzi et al., 2010).

It has been postulated that ROS are important players in degenerative diseases (Emerit, Edeas, & Bricaire, 2004). ROS generate protein and lipid oxidations, DNA damage and induce the death of neuronal cells (Wang et al., 2014). The neurotoxic effect of Aβ is associated with the production of ROS. Recent studies showed that cyanidin and malvidin 3-glucoside have protective effects against Aβ-induced neurotoxicity through inhibiting ROS formation (Shih, Wu, Yeh, & Yen, 2011; Thummayot et al., 2014).

In this study, we evaluated the effect of G13F and A13F extracts on ROS production by measuring intracellular ROS levels with the

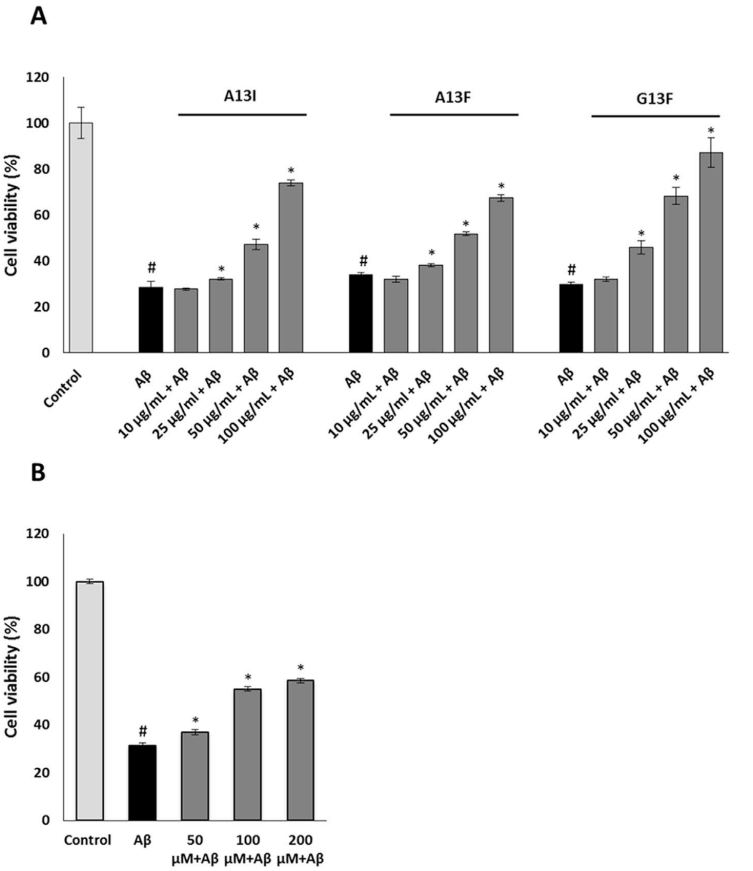

Fig. 2. (A–B). Cells viability determination. Cells were treated 24 h by strawberry extracts (A) or pelargonidin 3-glucoside (B), in presence or absence of 10 μM of $A\beta_{25\text{-}35}$. Results are expressed as mean SEM of four replicates (n = 4). $^{\sharp}$ P < 0.05 $A\beta_{(25\text{-}35)}$ versus control, *P < 0.05 extract versus $A\beta_{(25\text{-}35)}$.

DCFH-DA assay. As shown in Fig. 3, $A\beta_{25\text{-}35}$ treatment for 6 h induced a 1.8- to 2.4-fold increase in ROS production. Co-treatment with different concentrations of strawberry extracts significantly attenuated intracellular ROS accumulation. Strawberry extracts

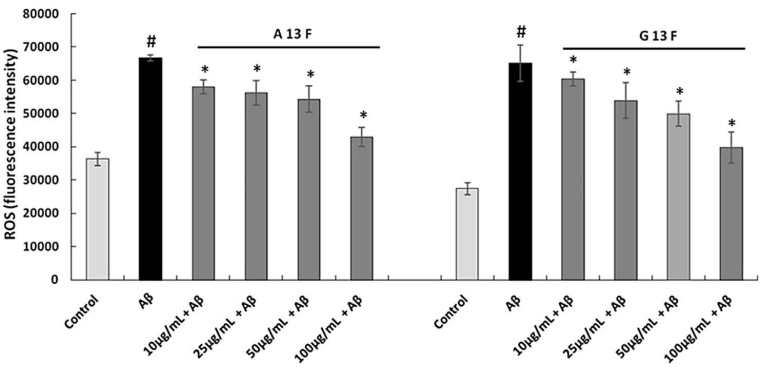

Fig. 3. ROS (Fluorescence intensity). Production of ROS in PC12. Cells were treated for 6 h by alcoholic and gluconic strawberry extracts, in presence or absence of 20 μM of $A\beta_{(25\text{-}35)}$. Results are expressed as mean SEM of four replicates (n = 4). ♯ P < 0.05 $A\beta_{(25\text{-}35)}$ versus control, *P < 0.05 extract versus $A\beta_{(25\text{-}35)}$.

decreased the production of ROS in a dose-dependent manner that can attenuate Aβ-induced oxidative stress proving this specific antioxidant activity.

4. Conclusion

Alcoholic fermentation of strawberry pureés decreased the anthocyanin content while gluconic fermentation preserved these compounds, which is an advantage of this last process. All these extracts were protective against Aβ-peptide neurotoxicity in PC12 cells, with gluconic fermented strawberry extracts being the most effective, as determined by cell viability and intracellular ROS production. Taken together, our experiments suggest that the fermented derivatives of strawberry are a good source of antioxidant bioactives.

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Influence of storage conditions on the anthocyanin profile and colour of an innovative beverage elaborated by gluconic fermentation of strawberry

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ABSTRACT

Derived fruit products such as strawberry-based fermented beverages, increase the fruit's conservation period and offer new alternatives for the non-alcoholic market. The influence of storage time and temperature on the anthocyanin composition, antioxidant activity and colour of a fermented strawberry beverage were studied and indicated that 60-days is the half-life for the tested beverage. Twenty-three anthocyanin compounds were analysed by ultra high-performance liquid chromatography Orbitrap (UHPLC-MS/MS). This is the first time that pelargonidin 3-sambubioside, delphinidin 3-arabinoside, cyanidin 3-(6-acetyl)-glucoside and delphinidin 3-galactoside have been reported in any products derived from strawberry. Additionally, the accurate mass of the following anthocyanins were reported: catechin-(4–8)-pelargonidin 3-glucoside, afzelechin-pelargonidin 3-glucoside, pelargonidin dissacharide-(hexose + pentose)-acylated with acetic acid, 5-carboxypyranopelargonidin 3-glucoside, pelargonidin 3-arabinoside, pelargonidin 3-malonylglucoside, pelargonidin 3-(6-acetyl)-glucoside and pelargonidin 3-(6-succynil)-arabinoside. Functional aspects of this drink rely on its bioactive compounds and lack of glucose due to its transformation to gluconic acid which makes it suitable for diabetic consumers.

Abbreviations: UHPLC-MS/MS, ultra high-performance liquid chromatography; eNOS, endothelial nitric oxide synthase; NO, nitric oxide; ORAC, oxygen radical absorbance capacity; DPPH, 2,2-diphenyl-1-picrylhydrazyl

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Chemical compounds: Pelargonidin 3-glucoside (PubChem CID: 443448); Pelargonidin 3-sambubioside (PubChem CID: 71627264); Pelargonidin 3-rutinoside (PubChem CID: 44256626); Pelargonidin 3-arabinoside (PubChem CID: 44256694); Pelargonidin 3- (6"-malonylglucoside) (PubChem CID: 45256635); Cyanidin 3-galactoside (PubChem CID: 44256700); Cyanidin 3-glucoside (PubChem CID: 92131208); Peonidin 3-glucoside (PubChem CID: 443654); Delphinidin 3-arabinoside (PubChem CID: 12137508); Delphinidin 3-glucoside (PubChem CID: 443650).

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1. Introduction

Strawberry [*Fragaria x ananassa* (*Rosaceae* Family)] is harvested in a very short period of time and is a very perishable product leading to the fruit rotting. The fruit has to be discarded if not sold, which entails substantial economic loss. Consequently, manufacturing derived products, such as fermented beverages using strawberries as a raw material, increases the conservation period and offers new sustainable and successful alternatives for the strawberry market, which drives economic profit.

Strawberry is a good source of nutrients, phytochemicals and fibre. The anthocyanins are among the principal bioactive compounds in strawberry (Basu, Nguyen, Betts, & Lyons, 2014). Additionally, these compounds are crucial for its colour quality. Several investigations have revealed antioxidant, anti-inflammatory, antihypertensive and anti-hyperlipidaemic or antiproliferative effects of strawberry anthocyanin compounds (Basu et al., 2014). Reduction on the risk of hypertension (8%) was observed when the consumption of strawberry anthocyanin was between 16 and 22 mg/day compared with a lower consumption (5–7 mg/day of anthocyanins) (Cassidy et al., 2011). Pure cyanidin 3-glucoside, induced endothelial nitric oxide synthase (eNOS) expression and increased nitric oxide (NO) release, which may help ameliorating endothelial dysfunction and maintain the blood pressure (Xu, Ikeda, & Yamori, 2004). Antioxidant and antiproliferative activities *in vitro* have been reported by Zhang, Seeram, Lee, Feng, and Heber (2008) when evaluating purified anthocyanin compounds in different human cancer cells (oral, colon and prostate).

It is well known that pelargonidin 3-glucoside is the major anthocyanin in strawberry (153–652 mg/kg fresh weight) followed by pelargonidin 3-rutinoside and other pelargonidin and cyanidin derivatives (Cerezo, Cuevas, Winterhalter, García-Parrilla, & Troncoso, 2010a; Lopes-da-Silva, Escribano-Bailon, Perez Alonso, Rivas-Gonzalo, & Santos-Buelga, 2007).

Recently, delphinidin 3-glucoside, peonidin 3-glucoside, and cyanidin 3-galactoside were identified in strawberry for the first time (Cerezo et al., 2010a). Other minor anthocyanin compounds, such as 5-carboxypyranopelargonidin 3-glucoside, pelargonidin acylated derivatives and pelargonidin linked to flavanol, were also present in strawberry (Fossen, Rayyan, & Andersen, 2004; Lopes-da-Silva, de Pascual-Teresa, Rivas-Gonzalo, & Santos-Buelga, 2002; Lopes-da-Silva et al., 2007).

Different types of processes are used to obtain more appropriate and attractive strawberry derivative products. The anthocyanin composition of processed products from berries such as liqueurs, juices, nectar, purée, condiments and jams has been studied only in terms of total anthocyanins (Da Silva, Lajolo, & Genovese, 2007; Klopotek, Otto, & Böhm, 2005; Sokół-Łętowska et al., 2014; Ubeda et al., 2013). However, there are no previous studies involving characterization of anthocyanin profile of strawberry fermented products, being restricted to non-anthocyanin composition (Álvarez-Fernández, Hornedo-Ortega, Cerezo, Troncoso, & García-Parrilla, 2014).

Process, time, and storage temperature are crucial factors that influence significantly the stability of anthocyanin compounds (Clifford, 2000). Tiwari, O'Donnel, Patras, Brunton, and Cullen (2009) observed greater stability of pelargonidin 3-glucoside and antioxidant activity at 4 °C compared to 20 °C in strawberry juice. Furthermore, anthocyanin compounds in liqueurs made from red fruits have been preserved for 3 months at 15 °C (Sokół-Łętowska et al., 2014). Therefore, the stability of anthocyanin compounds in each food matrix might be different. Indeed alcoholic and acetic fermentation exert a different impact on anthocyanin profile. Effects of alcoholic fermentation have been extensively studied. During red wine fermentation, the monomeric anthocyanins undergo several reactions and associations leading to the formation of anthocyanin-derived pigments. These reactions include self-association and co-pigmentation, such as the formation of polymeric anthocyanins with flavan-3-ols and proanthocyanidins, as well as the formation of new pigments, such as pyranoanthocyanins (Brouillard, Chassaing, & Fougerousse, 2003; Jackson, 2008; Wrolstad, Durst, & Lee, 2005). Acetification process of red wine also modifies anthocyanin composition, increasing vitisin-type and ethyl-linked compounds and decreasing the monomeric anthocyanins (Cerezo, Cuevas, Winterhalter, Garcia-Parrilla, & Troncoso, 2010b). However, the impact to anthocyanin composition of the gluconic fermented matrix products has not been studied.

These beverages represent an innovative trend as they lack glucose and therefore can be consumed by diabetics who can benefit from the health effects of anthocyanin.

The aim of this study was to examine the effect of storage time and temperature on the bioactive compounds (anthocyanins), antioxidant activity and colour, as this sensory parameters influence consumer acceptability of a strawberry beverage obtained through gluconic fermentation. UHPLC coupled with a hybrid mass spectrometer, which combined a linear trap quadrupole (LQT) and an Orbitrap mass analyser, was used to identify and quantify the anthocyanin compounds. Finally, the antioxidant activity of the extracts was evaluated using oxygen radical absorbance capacity (ORAC) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) essays; their colour parameters were also studied.

2. Materials and methods

2.1. Chemicals and materials

Amberlite XAD7HP, DPPH, 2,2′-diazo-bis-amidinepropane-dihydrochloride (AAPH), and Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic) were purchased from Sigma (Steinheim, Germany). Fluorescein sodium was obtained by Fluka (Steinheim, Germany). Acetonitrile was obtained from Merck (Darmstadt, Germany) and formic acid was obtained from Panreac (Barcelona, Spain).

Pelargonidin 3-glucoside, cyanidin 3-glucoside, delphinidin 3-glucoside and peonidin 3-glucoside were purchased from Chromadex Inc. (Irvine, CA, USA).

2.2. Gluconic fermented strawberry beverage

The strawberry beverage was obtained by gluconic fermentation previously described (Álvarez-Fernández et al., 2014). The process is detailed below: strawberry purées were the sub-

strate for fermentation and provided by Hudisa Company (Lepe, Huelva, Spain). *Gluconobacter japonicus* was used as a starter for the fermentation process. The fermentation was carried out by submerged culture. Three litres of strawberry purée substrate were placed into the bioreactor and the conditions set (29 °C, 20% O_2 and 1250 g); after 10–20 min, 125 mL of inoculum of *G. japonicus* strain E1 were added and mixed for 20–30 min. The process starts as the strawberry purée is mixed with the inoculum (strawberry substrate before fermentation) and finishes as the remaining glucose is totally consumed, then the fermentor is discharged. Subsequently, this product is immediately pasteurized by heating at 70–80 °C for 15 min and then rapidly cooled and frozen. These fermented strawberry products were supplied by the Department of Analytical Chemistry, Chemical Engineering, Faculty of Sciences, University of Cordoba (Cordoba, Spain).

Finally, the beverage (Samples 0) was formulated according to the following procedure. The fermented product obtained as describe before was centrifuged (1500 g) and the supernatant was collected and mixed with carbonated water (80:20, v/v). A few gouts of Stevia® were added to increase sweetness to the final product. We divided this product into different aliquots for the purpose of this study. Aliquots were stored at different temperatures (4 and 25 °C) and samples were taken at different times (0, 15, 30, 60 and 90 days). Sample codes indicate: initial (0), samples at room temperature at 15, 30, 60 and 90 days (R15, R30, R60 and R90) and samples at fridge temperature at 15, 30, 60 and 90 days (F15, F30, F60 and F90).

2.3. *Sample preparation*

The method was carried out according Cerezo et al. (2010a). An Amberlite XAD7HP column (30 × 1.5 cm) was conditioned with 200 mL of methanol and then 200 mL of water. Subsequently, a total of 20–30 mL of sample were loaded onto the column and cleaned with water to eliminate sugars, acids and polar compounds. Later, the anthocyanin fraction was eluted with a mixture of methanol/acetic acid (19:1, v/v); flow rate 1 drop/s. This fraction was collected and concentrated with a rotary evaporator under vacuum (Büchi Rotavapor, Flawil, Switzerland). The extracts were reconstituted in 2 mL of acidified water (5% formic acid) and stored at –20 °C until analysis.

2.4. *UHPLC-MS/MS Orbitrap analysis*

All analyses were performed using a liquid chromatography system consisting of a quaternary Rs Pump Dionex Ultimate 3000 (serial number: 8077352) and Rs autosampler Dionex Ultimate 3000 (serial number 8077399), connected to a quadrupole-orbitral (Qexactive) hybrid mass spectrometer with heated-electrospray ionization probe (HESI-II, Thermo Fisher Scientific, Bremen, Germany). Xcalibur software (version 3.0.63) was used for instrument control, data acquisition and data analysis. The analytical method was previously published (Natić et al., 2015). Separation was performed on a column SB-C18 (2.1 × 100 mm, 1.8 μm) (Agilent, USA). Injection volume was 1 μL and flow rate was 0.4 mL/min. Two different solvents were used as a mobile phase: Solvent A (Water/formic acid 95:5, v/v) and solvent B (acetonitrile/formic acid 95:5, v/v), scheduled in the following gradient: 0.0–2.0 min 5% B, 2.0–12.0 min from 5 to 100% B, 12.0–13.0 min from 100 to 5% B, then 5% B up to 15.0 min. Anthocyanin identification and quantification were acquired in positive mode by full-range acquisition covering *m/z* 100–1500 at 35,000 resolution and by targeted MS^2 normalized Higher Energy Collision Dissociation (HCD). Compounds were identified according to their mass spectra, calculated mass, characteristic fragmentation and retention time. Above mentioned available standards were used both for identification and quantification purposes. HESI source parameters were as follows: cell at 20 eV, source voltage 3.5 kV tube lens voltage 50 V, capillary temperature 263 °C, and sheath and auxiliary gas flow rate (N_2) 50 and 13 (arbitrary units).

2.5. *Antioxidant activity*

2.5.1. *DPPH method*

Scavenging activity against the stable coloured DPPH free-radical was assayed according to Katalinic, Milos, Kulisic, and Jukic (2006) with slight modifications, using 96-well plates. A total of 50 μL of extracts (1–15 μL/mL) were added to each well and completed to a final volume of 200 μL with 150 μM of methanolic solution of DPPH. Plates were shaken for 10 s and then absorbance was read after 20 min in a multi-detector microplate reader at 517 nm (Synergy HT, Biotek®, Winooski, Vermont, USA). The inhibition percentage of DPPH radical (1%) was calculated as follows:

$$1\% = ((A_C - A_S)/As) \times 100 \quad (1)$$

Where A_C is absorbance of the control without sample and A_S is absorbance of the tested sample measured at 20 min.

Sample concentration providing 50% inhibition (IC_{50}) was calculated from the graph, plotting inhibition percentage against sample concentration (μL/mL). All experiments were performed in triplicate.

2.5.2. *ORAC test*

The procedure is based on a previously reported method (Ou, Hampsch-Woodill, & Prior, 2001) with slight modifications. Fifty microlitres of sample or Trolox were mixed with 100 μL of fluorescein (45 mM) and 50 μL of AAPH (15 mM). Fluorescence was recorded for 90 min (excitation wavelength was set at 485 nm and emission wavelength at 528 nm). Measurements were taken in triplicate in a multi-detector microplate reader (Synergy HT, Biotek®). Trolox was used as a calibration standard in the 0.5–9.5 μM concentration range previously established (Cerezo et al., 2010a).

Fluorescein fluorescence was recorded every 5 min after addition of AAPH until fluorescence was <5% of the initial reading. Final results were calculated using the areas under the fluorescein decay curves between the blank and the sample and were expressed as millimol Trolox equivalent/mL beverage.

2.6. *Determination of colour stability*

Colour measurements were determined using a Konica Minolta CM-3600d spectrophotometer (Minolta Co. Ltd., Osaka, Japan) in the CIELab colour space, with the D65 illuminant and 10° observer. The colour coordinates of the beverages were com-

puted in the CIELAB scale in a CIE D65/10° illuminant/observer condition. Colour results were expressed as tristimulus parameters (L^*, a^*, b^*, H^*, C^*). Hue angle

$$\left(H^* = \tan^{-1} b^*/a^*\right) \quad (2)$$

indicates sample colour (0° or 360° = red, 90° = yellow, 180° = green, 270° = blue), and chroma

$$\left(C^* = \left[a^{*2} + b^{*2}\right]^{1/2}\right) \quad (3)$$

indicates colour purity or saturation (high values are more vivid); a^* and b^* chromaticity coordinates indicate colour directions green (–a^*)/red (+a^*) and blue (–b^*)/yellow (+b^*) (Bakker, Bridle, & Timberlake, 1986). Three measurements were taken for each sample.

2.7. Statistical analysis

One-way analysis of variance (ANOVA test) ($p < 0.005$) was used to explore significant differences in anthocyanin composition between sample 0 and the other sample times, using Statistica software (StatSoft Inc, 2004). The IC_{50} was calculated by Graphpad Prism 6 software.

3. Results and discussion

3.1. Identification of anthocyanin compounds by UHPLC-MS/MS Orbitrap

Tables 1 and 2 summarize the retention times (min), molecular formula (M^+), calculated mass and accurate mass, (m/z), accuracy error (ppm), and MS/MS fragments (accurate mass (m/z), % fragments, calculated mass (m/z), molecular formula, error (ppm)) of a total of 23 compounds identified by UHPLC-MS/MS Orbitrap either matching with available standards or data reported in the literature (tentative identification) when standards were not commercially available.

The anthocyanin profile of strawberry beverages and strawberry purée includes a total of 15 pelargonidin derivates, 4 cyanidin derivates, 3 delphinidin derivates and 1 peonidin derivate (Fig. 1). It is the first time that pelargonidin 3-sambubioside, delphinidin 3-arabinoside, cyanidin 3-(6-acetyl)-glucoside and delphinidin 3-galactoside have been tentatively identified in strawberry or a product derived from strawberry. Furthermore, the calculated masses of catechin-(4–8)-pelargonidin 3-glucoside, afzelechin-pelargonidin 3-glucoside, pelargonidin dissacharide (hexose + pentose) acylated with acetic acid, 5-carboxypyranopelargonidin 3-glucoside, pelargonidin 3-arabinoside, pelargonidin 3-malonylglucoside, pelargonidin 3-(6-acetyl)-glucoside and pelargonidin 3-(6-succynil)-arabinoside/ 3-(6-malonyl)-rhamnoside (peaks 1, 2, 11,12, 15, 17, 20 and 23, respectively) are reported as an original contribution of this study (Table 1).

Pelargonidin 3-glucoside (peak 8) was the major compound, followed by pelargonidin 3-rutinoside (peak 9) as showed the positive identification and quantification with standards, in accordance with the literature (Lopes-da-Silva et al., 2002).

Additionally, other pelargonidin derivatives linked to flavonols were identified as follows: catechin-(4–8)-pelargonidin 3-glucoside (peak 1), afzelechin-pelargonidin 3-glucoside and Epi-afzelechin-pelargonidin 3-glucoside (peak 2 and 4, respectively). The latter presented identical accurate masses 705.1796/705.1801 and fragmentation patterns (543/407/313), but different retention times, due to their stereochemistry, which influences overall polarity. Moreover, peaks 11, 14 and 16 correspond to pelargonidin dissacharide (hexose + pentose) acylated with acetic acid. All of them presented the same accurate mass and fragment (*m/z* 271) but different retention times. This might be explained by the presence of different sugar substituents or by a different linkage occurring between the pentose and hexose residues in each of the anomeric carbons of the pigments. For the first time, this contribution reports the tentative identification of pelargonidin 3-sambubioside (peak 7) in a strawberry derivative, with an accurate mass of m/z 565.1552 with –1.3499 ppm of error (Fig. 2A). This compound has been identified in other berries, specifically in raspberry extract, with similar high resolution instrumentation (m/z 565.1554) (Mullen, Larcombe, Arnold, Welchman, & Crozier, 2010).

Concerning cyanidin derivates, we identified cyanidin 3-galactoside, cyanidin 3-rutinoside and cyanidin 3-glucoside, all of which have also been detected by Lopes-da-Silva et al. (2007) and Cerezo et al. (2010a) (peaks 5, 6 and 21, respectively).

In addition, the other novelty of this contribution is the tentative identification of cyanidin 3-(6-acetyl)-glucoside (peak 18) (Fig. 2B). This compound has been previously described by Brito, Areche, Sepúlveda, Kennelly, and Simirgioti (2014) in six Chilean berry extracts by HR-ToF-ESI-MS. They found cyanidin 3-O-(6"acetyl)-glucoside in blueberries (*Vaccinium corymbosum*) and berries (*Berberis microphylla*) with an HR-M^+ ion of 491.1206. In addition, this compound has been identified in the skin and wine of the grape Vitis vinifera L. (cv. Aglianico) (De Nisco et al., 2013).

Three derivatives of delphinidin were identified: delphinidin 3-glucoside (peak 22), which was previously reported for the first time by our group in *Camarosa* strawberry purée (Cerezo et al., 2010a); delphinidin 3-arabinoside (m/z 435.0910 and fragment 303) and delphinidin 3-galactoside (peaks 13 and 19, respectively), which have not been described in any strawberry derivative product before (Figs 3 and 4). This anthocyanin compound has been previously identified in other berries (blueberry, blackcurrant and Chilean berry) (Brito et al., 2014; Mullen et al., 2010; Sokół-Łętowska et al., 2014).

We could state that all these compounds are naturally present in the strawberry purée and their occurrence in the beverage is not due to the fermentation process (Table 1).

3.2. Effects of temperature on anthocyanin concentration during storage

All the anthocyanin compounds were quantified using the areas of the aglycone counterparts.

Tables 2 and 3 display anthocyanin concentrations of strawberry substrate and gluconic fermented beverages, respectively. Comparing the strawberry substrate sample (before fermentation) with initial sample (0), we observed that the majority of anthocyanin compounds decreased significantly ($p < 0.05$). Fermentation process affects severely the anthocyanin com-

Table 1 – Anthocyanins found in strawberry substrate before fermentation, initial sample and strawberry beverage samples in positive ionization mode. Mean expected retention time (Rt) (min), molecular formula, calculated mass (*m/z*), accurate mass (*m/z*), accuracy error (ppm) and MS/MS fragments (Accurate mass (*m/z*), % fragments, calculated mass (m/z), molecular formula, error (ppm %)).

Peak	Rt (min)	Tentatively identification	Molecular formula (M^+)	Calculated mass (*m/z*)	Accurate mass (*m/z*)	Error (ppm)	MS/MS fragments (Accurate mass (*m/z*), % fragments, calculated mass (*m/z*), molecular formula, error (ppm))	References
1	4.07	Catechin-(4–8)-pelargonidin 3-glucoside	$C_{36}H_{33}O_{16}^+$	721.1763[b]	721.1744	−2.6814	559.1226 (82.31) / 559.1226/ $C_{30}H_{23}O_{11}^+$/ −1.6621 407.0753 (10.26) / 407.0761/ $C_{22}H_{15}O_8^+$/ −1.9553 313.0698 (7.43) / 313.0707/ $C_{17}H_{13}O_6^+$/ −2.6843	Fossen et al. (2004); Cerezo et al. (2010a)
2	4.61	Epi-Afzelechin-pelargonidin 3- glucoside	$C_{36}H_{33}O_{15}^+$	705.1814[b]	705.1795	−2.6833	543.1277(79.49) / 543.1286/ $C_{30}H_{23}O_{10}^+$/ −1.4365 407.0750 (11.69) / 407.0761/ $C_{22}H_{15}O_8^+$/ −2.7799 313.0698 (8.82) / 313.0707/ $C_{17}H_{13}O_6^+$/ −2.8793	Fossen et al. (2004); Cerezo et al. (2010a)
3	4.61	Pelargonidin 3,5-diglucoside	$C_{27}H_{31}O_{15}^+$	595.1657	595.1641	−2.8295	433.1135 (52.82) / 433.1129/ $C_{21}H_{21}O_{10}^+$/ 1.3198 271.0592 (47.18) / 271.0601/ $C_{15}H_{11}O_5^+$/ −3.4175	Lopes-da-Silva et al. (2002 and 2007); Cerezo et al. (2010a)
4	4.76	Epi-Afzelechin-pelargonidin 3- glucoside	$C_{36}H_{33}O_{15}^+$	705.1814[b]	705.1798	−2.2505	543.1277 (79.53) / 543.1286/ $C_{30}H_{23}O_{10}^+$/ −1.5221 407.0750 (11.72) / 407.0761/ $C_{22}H_{15}O_8^+$/ 2.7050 313.0698 (8.75) / 313.0698/ $C_{17}H_{13}O_6^+$/ −2.8793	Fossen et al. (2004); Cerezo et al. (2010a)
5	4.80	Cyanidin 3-galactoside	$C_{21}H_{21}O_{11}^+$	449.1078	449.1074	−0.9941	287.0544 (100) / 287.0550/ $C_{15}H_{11}O_6^+$/ −2.2024	Cerezo et al. (2010a)
6	4.84	Cyanidin 3-rutinoside	$C_{27}H_{31}O_{15}^+$	595.1657	595.1661	0.5547	449.1075 (5.06) / 449.1078/ $C_{21}H_{21}O_{11}^+$/ −0.7544 287.0547 (94.94) / 287.0550/ $C_{15}H_{11}O_6^+$/ −1.1393	Lopes-da-Silva et al. (2002); Wu and Prior (2005); Cerezo et al. (2010a)
7	4.94	Pelargonidin 3-sambubioside	$C_{26}H_{29}O_{14}^+$	565.1552	565.1544	−1.3499	271.0595 (100) / 271.0601/ $C_{15}H_{11}O_5^+$/ −2.2917	[a]
8	4.96	Pelargonidin 3- glucoside[c]	$C_{21}H_{21}O_{10}^+$	433.1129	433.1122	−1.7804	271.0596 (100) / 271.0601/ $C_{15}H_{11}O_5^+$/ −1.7287	Lopes-da-Silva et al. (2002); Wu and Prior (2005); Cerezo et al. (2010a)
9	5.08	Pelargonidin 3- rutinoside	$C_{27}H_{31}O_{14}^+$	579.1708	579.1702	−1.1498	433.1118 (4.46) / 433.1129/ $C_{21}H_{21}O_{10}^+$/ −2.5555 271.0594 (95.54) / 271.0601/ $C_{15}H_{11}O_5^+$/ −2.4042	Lopes-da-Silva et al. (2002); Wu and Prior (2005); Cerezo et al. (2010a)
10	5.15	Peonidin 3-glucoside[c]	$C_{22}H_{23}O_{11}^+$	463.1235	463.1230	−1.0841	301.0699 (100) / 301.0707/ $C_{16}H_{13}O_6^+$/ −2.6899	Cerezo et al. (2010a)
11	5.16	Pelargonidin dissacharide (hexose + pentose) acylated with acetic acid	$C_{28}H_{31}O_{15}^+$	607.1657[b]	607.1650	−1.2657	271.0597 (100) / 271.0601/ $C_{15}H_{11}O_5^+$/ −1.6161	Lopes-da-Silva et al. (2002); Cerezo et al. (2010a)
12	5.19	5-carboxypyranopelargonidin 3-glucoside	$C_{24}H_{21}O_{12}^+$	501.1028[b]	501.1018	−1.8262	339.0493 (100) / 339.0499/ $C_{18}H_{11}O_7^+$ / −1.9872	Lopes-da-Silva et al. (2007); Cerezo et al. (2010a)
13	5.28	Delphinidin 3-arabinoside	$C_{20}H_{19}O_{11}^+$	435.0922	435.0909	−2.8624	303.0488 (100) / 303.0499/ $C_{15}H_{11}O_7^+$/ −3.6282	[a]
14	5.31	Pelargonidin dissacharide (hexose + pentose) acylated with acetic acid	$C_{28}H_{31}O_{15}^+$	607.1657[b]	607.1657	−0.0594	271.0593 (100) / 271.0601/ $C_{15}H_{11}O_5^+$/ −2.9672	Lopes-da-Silva et al. (2002); Cerezo et al. (2010a)
15	5.40	Pelargonidin 3-arabinoside	$C_{20}H_{19}O_9^+$	403.1024[b]	403.1719	−1.2935	271.0598 (100) / 271.0601/ $C_{15}H_{11}O_5^+$/ −0.9406	Lopes-da-Silva et al. (2007)
16	5.42	Pelargonidin dissacharide (hexose + pentose) acylated with acetic acid	$C_{28}H_{31}O_{15}^+$	607.1657[b]	607.1649	−1.4668	271.0592 (100) / 271.0601/ $C_{15}H_{11}O_5^+$/ −3.4175	Lopes-da-Silva et al. (2002); Cerezo et al. (2010a)
17	5.47	Pelargonidin 3-malonylglucoside	$C_{24}H_{23}O_{13}^+$	519.1133[b]	519.1125	−1.4800	433.1115 (0.39) / 433.1129/ $C_{21}H_{21}O_{10}^+$/ −3.4011 271.0596 (99.61) / 271.0601/ $C_{15}H_{11}O_5^+$/ −1.8413	Lopes-da-Silva et al. (2007)
18	5.56	Cyanidin 3-(6-acetyl)-glucoside	$C_{23}H_{23}O_{12}^+$	491.1184	491.1180	−0.7962	287.0542 (100) / 287.0550/ $C_{15}H_{11}O_6^+$/ −2.8403	[a]
19	5.68	Delphinidin 3-galactoside	$C_{21}H_{21}O_{12}^+$	465.1028	465.1028	0.1829	303.0488 (100) /303.0499/ $C_{15}H_{11}O_7^+$/ −3.6328	[a]
20	5.81	Pelargonidin 3-(6-acetyl)-glucoside	$C_{23}H_{23}O_{11}^+$	475.1235[b]	475.1227	−1.6348	271.0597 (100) / 271.0601/ $C_{15}H_{11}O_5^+$/ −1.3909	Lopes-da-Silva et al. (2007); Cerezo et al. (2010a)
21	5.92	Cyanidin 3-glucoside[c]	$C_{21}H_{21}O_{11}^+$	449.1078	449.1068	−2.2173	287.0544 (100) / 287.0550/ $C_{15}H_{11}O_6^+$/ −2.3087	Lopes-da-Silva et al. (2002); Wu and Prior (2005); Cerezo et al. (2010a)
22	6.27	Delphinidin 3-glucoside[c]	$C_{21}H_{21}O_{12}^+$	465.1028[b]	465.1028	0.1054	303.0491 (100) / 303.0499/ $C_{15}H_{11}O_7^+$/ −2.7661	Cerezo et al. (2010a)
23	6.35	Pelargonidin 3-(6-succinyl)-arabinoside/ 3-(6-malonyl)-rhamnoside	$C_{25}H_{27}O_{11}^+$	503.1548	503.1537	−2.0679	271.0596 (100) / 271.0601/ $C_{15}H_{11}O_5^+$/ −1.3909	Aaby, Ekeberg, and Skrede (2007); Cerezo et al. (2010a)

[a] Non-previously reported in strawberry.
[b] Exact mass reported for the first time in strawberry and strawberry derivate products.
[c] Compounds identified with commercial standards.

Table 2 – Concentration (μg/L) of anthocyanins present in strawberry substrate before fermentation, initial sample and samples stored at room temperature samples.

Peak	Compound	Strawberry substrate	0	R15	R30	R60	R90
1	Catechin-(4–8)-pelargonidin 3-glucoside	9.31 ± 0.00	13.52 ± 0.08[a]	9.55 ± 0.00[b]	7.95 ± 0.06[c]	2.86 ± 0.01[d]	1.45 ± 0.01[e]
2	Epi-Afzelechin-pelargonidin 3-glucoside	7.86 ± 0.00	8.10 ± 0.03[a]	4.89 ± 0.02[b]	3.92 ± 0.08[c]	0.30 ± 0.00[d]	0.09 ± 0.00[e]
3	Pelargonidin 3,5-diglucoside	0.40 ± 0.00	0.62 ± 0.00[a]	0.41 ± 0.01[b]	0.41 ± 0.06[c]	0.17 ± 0.01[d]	0.15 ± 0.02[e]
4	Epi-Afzelechin-pelargonidin 3-glucoside	2.14 ± 0.08	3.20 ± 0.08[a]	1.94 ± 0.07[b]	1.98 ± 0.04[c]	0.19 ± 0.01[d]	0.04 ± 0.00[e]
5	Cyanidin 3-galactoside	187.95 ± 0.09	61.48 ± 1.22[a]	31.38 ± 0.77[b]	21.64 ± 0.08[c]	4.73 ± 0.10[d]	0.60 ± 0.10[e]
6	Cyanidin 3-rutinoside	6.50 ± 0.07	3.61 ± 0.29[a]	2.50 ± 0.04[b]	1.96 ± 0.11[c]	0.79 ± 0.02[d]	0.48 ± 0.11[e]
7	Pelargonidin 3-sambubioside	1.11 ± 0.00	1.50 ± 0.11[a]	1.58 ± 0.03	1.22 ± 0.03[c]	0.10 ± 0.00[d]	0.06 ± 0.00[e]
8	Pelargonidin 3-glucoside	2113.59 ± 29.45	644.77 ± 4.24[a]	402.41 ± 13.5[b]	328.54 ± 5.38[c]	110.85 ± 14.43[d]	47.26 ± 1.54[e]
9	Pelargonidin 3-rutinoside	278.04 ± 0.95	62.13 ± 0.11[a]	50.01 ± 0.69[b]	42.66 ± 0.13[c]	13.48 ± 0.05[d]	6.79 ± 0.24[e]
10	Peonidin 3-glucoside	5.65 ± 0.04	1.10 ± 0.02[a]	0.66 ± 0.02[b]	0.53 ± 0.01[c]	0.20 ± 0.00[d]	0.19 ± 0.00[e]
11	Pelargonidin dissacharide (hexose + pentose) acylated with acetic acid	1.35 ± 0.01	1.40 ± 0.08[a]	0.78 ± 0.02[b]	0.11 ± 0.00[c]	0.07 ± 0.00[d]	0.06 ± 0.00[e]
12	5-carboxypyranopelargonidin 3-glucoside	66.31 ± 0.53	16.03 ± 0.65[a]	14.38 ± 1.24	18.15 ± 0.44[c]	17.56 ± 1.11	12.27 ± 0.43[e]
13	Delphinidin 3-arabinoside	2.60 ± 0.15	5.24 ± 0.08[a]	3.57 ± 0.01[b]	3.27 ± 0.04[c]	2.20 ± 0.01[d]	1.75 ± 0.21[e]
14	Pelargonidin dissacharide (hexose + pentose) acylated with acetic acid	0.88 ± 0.00	1.39 ± 0.02[a]	0.79 ± 0.00[b]	0.05 ± 0.00[c]	0.03 ± 0.00[d]	0.05 ± 0.00[e]
15	Pelargonidin 3-arabinoside	0.84 ± 0.00	0.62 ± 0.06[a]	0.30 ± 0.04[b]	0.24 ± 0.01[c]	0.03 ± 0.00[d]	0.09 ± 0.01[e]
16	Pelargonidin dissacharide (hexose + pentose) acylated with acetic acid	0.92 ± 0.00	0.61 ± 0.02[a]	0.34 ± 0.01[b]	0.35 ± 0.00[c]	0.20 ± 0.01[d]	0.13 ± 0.00[e]
17	Pelargonidin 3-malonylglucoside	124.90 ± 0.58	21.45 ± 0.45[a]	12.21 ± 0.71[b]	8.16 ± 0.73[c]	1.60 ± 0.08[d]	1.39 ± 0.02[e]
18	Cyanidin 3-(6-acetyl)-glucoside	1.83 ± 0.00	3.88 ± 0.05[a]	2.12 ± 0.02[b]	1.66 ± 0.22[c]	1.00 ± 0.02[d]	0.91 ± 0.00[e]
19	Delphinidin 3-galactoside	36.98 ± 0.01	20.94 ± 1.54[a]	15.20 ± 0.12[b]	14.80 ± 0.02[c]	14.60 ± 0.05[d]	15.17 ± 0.06[e]
20	Pelargonidin 3-(6-acetyl)-glucoside	149.75 ± 0.02	36.34 ± 0.13[a]	18.48 ± 0.16[b]	14.59 ± 0.12[c]	3.00 ± 0.02[d]	0.81 ± 0.02[e]
21	Cyanidin 3-glucoside	86.59 ± 0.01	41.02 ± 0.28[a]	29.19 ± 0.09[b]	27.05 ± 0.20[c]	27.79 ± 0.11[d]	26.85 ± 0.92[e]
22	Delphinidin 3-glucoside	1.05 ± 0.00	1.69 ± 0.07[a]	1.51 ± 0.03[b]	1.23 ± 0.02[c]	0.76 ± 0.04[d]	0.81 ± 0.01[e]
23	Pelargonidin 3-(6-succinyl)-arabinoside/ 3-(6-malonyl)-rhamnoside	32.86 ± 0.06	10.19 ± 0.10[a]	5.27 ± 0.02[b]	3.97 ± 0.17[c]	0.95 ± 0.07[d]	0.49 ± 0.00[e]

Different superscript letters mean significant differences ($p < 0.05$) between the samples as follows:
[a] Strawberry purée and 0;
[b] 0 and 15;
[c] 15 and 30;
[d] 30 and 60;
[e] 60 and 90.

position, decreasing their concentration approximately in a 70%. These results agree with previously published data regarding strawberry wine. Indeed, Klopotek et al. (2005) reported a decrease of 62.4 % after alcoholic fermentation of strawberry mash. An even larger decrease (90.2%) was determined after the fermentation of pomegranate (Ordoudi et al., 2014). Conversely, it must be highlighted that certain condensed anthocyanins and also some acetylated ones increased significantly. This is the case for catechin-(4–8)-pelargonidin 3-glucoside, both epi-afzelechin-pelargonidin 3-glucoside and pelargonidin dissacharide (hexose + pentose) acylated with acetic acid. These results are in accordance with Giusti and Wrolstad (2003) that reported that acylated compounds present a higher stability that monoacylated anthocyanins and they are likely formed during the fermentation process.

Regarding beverages, anthocyanin concentration decreased significantly ($p < 0.05$) after 15 days of storage at room temperature. The main compound pelargonidin 3-glucoside decreased significantly, by 37.7%, after 15 days of storage, and by 92.7% at 90 days. Pelargonidin 3-rutinoside and cyanidin 3-galactoside decreased by 89.1 and 99% at 90 days, respectively. A similar trend is shown for the other anthocyanin compounds. Conversely, the most stable compounds were cyanidin 3-glucoside, delphinidin 3-galactoside and 5-carboxypyranopelargonidin 3-glucoside, which decreased by only 34.5, 27.5, and 23.4%, respectively, at room temperature.

On the other hand, when samples were stored at 4 °C (fridge temperature), the concentrations of the main anthocyanin compounds were preserved until 60 days of storage, while minority compounds such as pelargonidin 3-(6-acetyl)-glucoside, pelargonidin 3-(6-succynil)-arabinoside, pelargonidin 3-sambubioside, cyanidin 3-(6-acetyl)-glucoside, cyanidin 3-glucoside and delphinidin 3-arabinoside diminished significantly after 15 days. The percentages of decrease of the major compounds were 34, 17.4 and 54.5% (pelargonidin 3-glucoside, pelargonidin 3-rutinoside and cyanidin 3-galactoside, respectively). The most stable compound was 5-carboxypyranopelargonidin 3-glucoside for both beverages samples (room and fridge temperature). In fact, Andersen, Fossen, Torskangerpoll, Fossen, and Hauge (2004) explained that the pyrano form restricted the formation of the unstable form and the colourless equilibrium in comparison with for ordi-

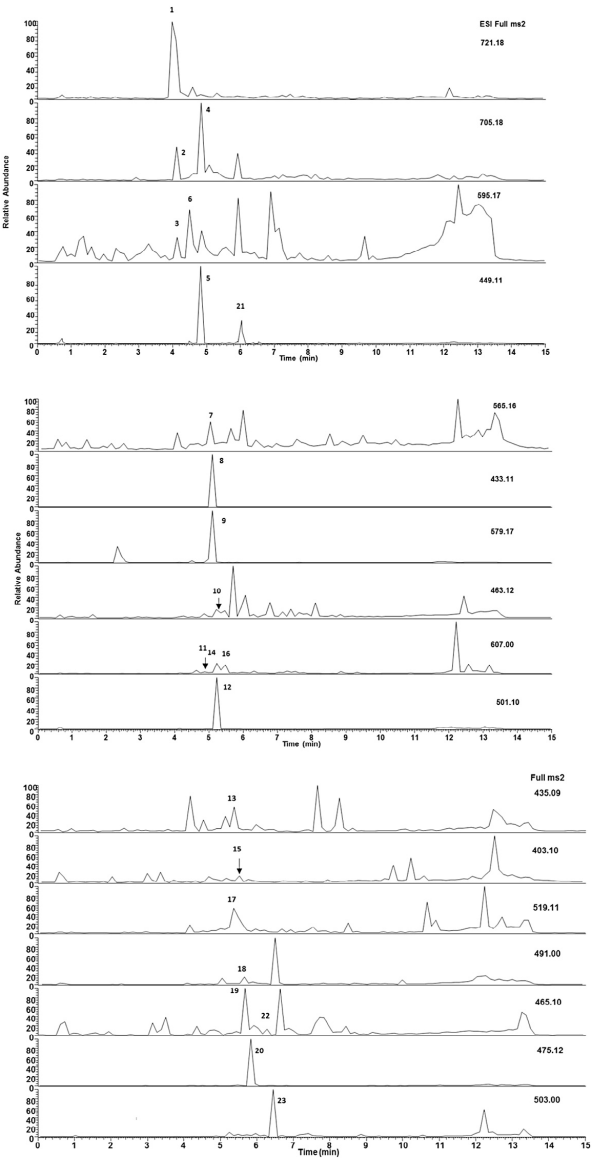

Fig. 1 – Ion chromatograms from targeted MS2 experiments selecting molecular ions from initial sample. 1: Catechin-(4–8)-pelargonidin 3-glucoside; 2: Afzelechin-pelargonidin 3-glucoside; 3: Pelargonidin 3,5-diglucoside; 4: Epi-Afzelechin-pelargonidin 3-glucoside; 5: Cyanidin 3-galactoside: 6: Cyanidin 3-rutinoside; 7: Pelargonidin 3-sambubioside; 8: Pelargonidin 3-glucoside; 9: Pelargonidin 3- rutinoside; 10: Peonidin 3-glucoside; 11: Pelargonidin dissacharide (hexose + pentose) acylated with acetic acid; 12: 5-carboxypyranopelargonidin 3-glucoside; 13: Delphinidin 3-arabinoside; 14: Pelargonidin dissacharide (hexose + pentose) acylated with acetic acid; 15: Pelargonidin 3-arabinoside; 16: Pelargonidin dissacharide (hexose + pentose) acylated with acetic acid; 17: Pelargonidin 3-malonylglucoside; 18: Cyanidin 3-(6-acetyl)-glucoside; 19: Delphinidin 3-galactoside; 20: Pelargonidin 3-(6-acetyl)-glucoside; 21: Cyanidin 3-glucoside; 22: Delphinidin 3-glucoside. 23: Pelargonidin 3-(6-succinyl)-arabinoside/ 3-(6-malonyl)-rhamnoside.

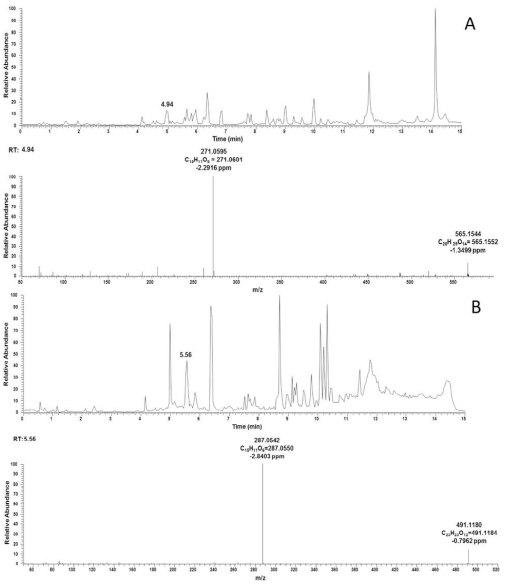

Fig. 2 – Extracted ion chromatogram (XIC) from targeted-MS2 experiment selecting *m/z* 565.1552 corresponding to pelargonidin 3-sambubioside of initial sample (0) (A upper) and MS2 spectra (A down). Extracted ion chromatogram (XIC) from targeted-MS2 experiment selecting *m/z* 491.1184 corresponding to cyanidin 3-(6-acetyl)–glucoside of initial sample (0) (B upper) and MS2 spectra (B down).

nary anthocyanins. We can conclude that temperature is crucial in conserving the beverage, with refrigeration being highly recommended to preserve anthocyanin compounds (Tables 2 and 3).

3.2.1. *pH, antioxidant activity and colour measurements*

Table 4 displays pH and antioxidant activity measured by the ORAC and DPPH and colour parameters of beverages in different storage conditions. The pH does not change under any of these conditions. During storage, in both temperature conditions, a decrease in antioxidant activity was observed for both tests: the higher the storage temperature, the greater the decrease in antioxidant activity. Specifically, in the ORAC test, diminution varies by 10% after 60 days at room temperature. For samples stored at fridge temperature, the diminution is less notable (5%).

The same trend was observed with the DPPH test. In order to achieve 50% DPPH inhibition, higher concentrations of the beverage were required when it was stored at room temperature. Similarly, Wiczkowski, Szawara-Nowak, and Topolska (2015), reported a 6% of decrease in ORAC value of fermented red cabbage after the storage at 4 °C during 90 days that is very close to our results (5.4%). Furthermore, the antioxidant activity of jams made from black carrot (*Daucus carota*) rich in anthocyanins compounds present a more acute decrease when stored at 4° and 20° ranging (8–54%) and (12.8–60.9%), respectively (Kamiloglu, Pasli, Ozcelik, Camp, & Capanoglu, 2015). Therefore, the pH = 2.5 that our beverage presents might account for the higher preservation of antioxidant capacity in our work. In fact, a pH = 2.5 has been reported to be the best condition for the preservation of strawberry anthocyanins after 90-day storage conditions (Oliveira et al., 2015).

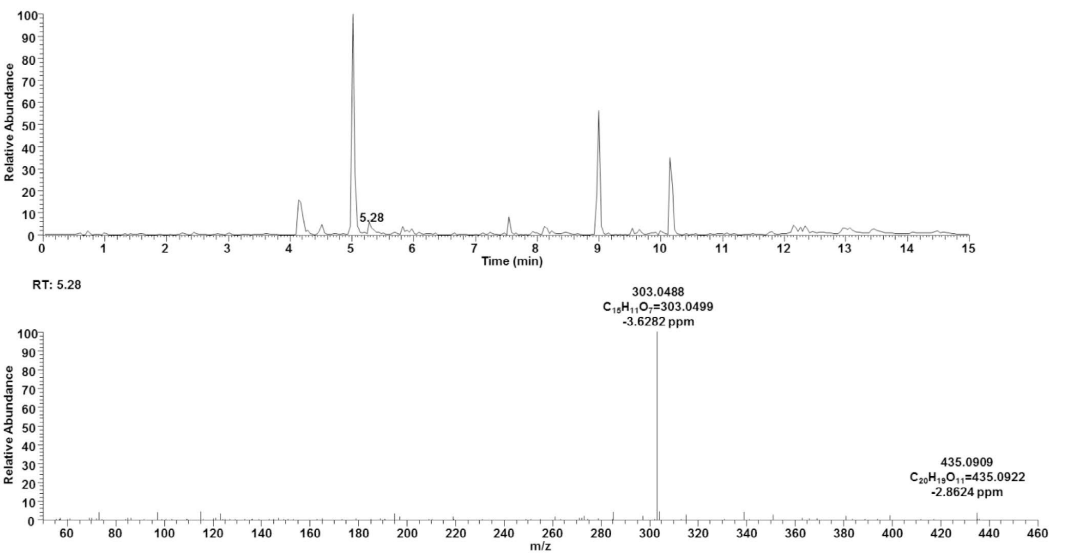

Fig. 3 – Extracted ion chromatogram (XIC) from targeted-MS2 experiment selecting *m/z* 435.0922 corresponding to delphinidin 3-arabinoside of initial sample (0) (upper) and MS2 spectra (down).

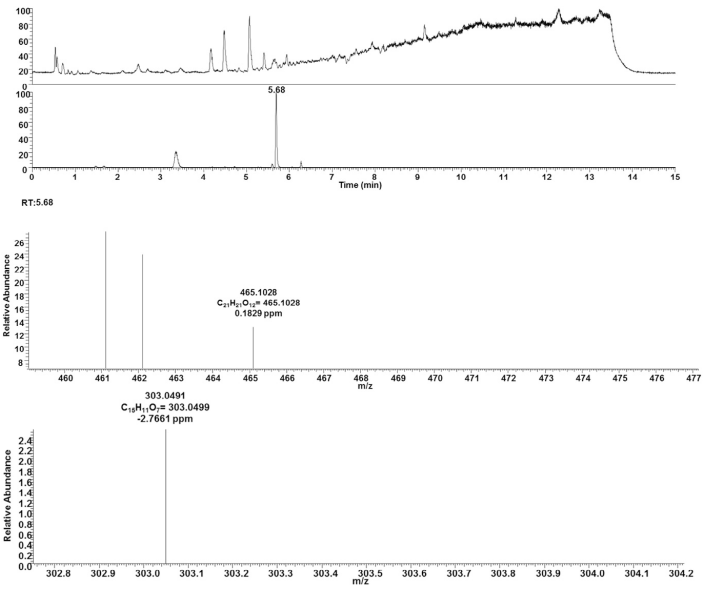

Fig. 4 – Extracted ion chromatogram (XIC) from full scan experiment selecting *m/z* 465.1028 corresponding to delphinidin 3-galactoside of initial sample (0) (upper) and MS2 spectra (down). Identified by full scan and confirmed with fragmentation pattern.

CIE Lab parameters of the strawberry beverage in different storage conditions were measured. The *L*, *b* and C values show statistical differences at 15 days, if samples are stored at room temperature. However, when samples are stored at 4 °C, the significant differences ($p < 0.05$) start at 30 days. In addition, for *a* and h values, we observed significant differences from 15 days at both temperatures. Apart from these values, Table 4 shows ΔE ("delta e"), which indicates the difference between two colours in an L*a*b* colour space. This parameter reflects whether the colour differences could be perceptible to the human eye and is calculated as follows:

$$\left(\Delta E = \sqrt{(\Delta L)^2 + (\Delta a)^2 + (\Delta b)^2}\right) \quad (4)$$

Where Δ*L*, Δ*a* and Δ*b* are the differences in the *L*, *a* and *b* values between initial and different storage condition samples.

The ΔE classification was based on the one reported by Cserhalmi, Sass-Kiss, Tóth-Markus, and Lechner (2006): 0–0.5 (not noticeable), 0.5–1.5 (slightly noticeable), 1.5–3.0 (noticeable), 3.0–6.0 (well visible) and >6.0 (great). Noticeable differences can be observed after 15 days of storage at room temperature. However, 60 days of storage at fridge temperature are required before appreciable differences can be perceived (4.871).

4. Conclusions

This paper reports for the first time anthocyanins mass with high accuracy (four decimals) of: catechin-(4–8)-pelargonidin 3-glucoside, afzelechin-pelargonidin 3-glucoside, pelargonidin dissacharide (hexose + pentose) acylated with acetic acid, 5-carboxypyranopelargonidin 3-glucoside, pelargonidin 3-arabinoside, pelargonidin 3-malonylglucoside, pelargonidin 3-(6-acetyl)-glucoside and pelargonidin 3-(6-succynil)-arabinoside. Additionally, this contribution reports the tentative identification of four compounds that have not been previously described in strawberry or products made from this fruit: pelargonidin 3-sambubioside, delphinidin 3-arabinoside, cyanidin 3-(6-acetyl)-glucoside and delphinidin3-galactoside based on calculated and accurated mass, error and fragmentation pattern data.

Storage temperature and time are crucial for conserving the beverage in terms of its anthocyanin profile and colour; consequently, refrigeration is highly recommended to preserve bioactive compounds. If the beverage is stored at 4 °C, sixty days is the beverage half-life for preserving its composition and properties.

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Table 3 – Concentration (μg/L) of anthocyanins present in strawberry substrate before fermentation, initial sample and samples stored at fridge temperature samples.

Peak	Compound	Strawberry substrate	0	F15	F30	F60	F90
1	Catechin-(4–8)-pelargonidin 3-glucoside	9.31 ± 0.00	13.52 ± 0.08[a]	12.20 ± 0.22[b]	12.57 ± 0.02	7.57 ± 0.07[d]	6.62 ± 0.12[e]
2	Epi-Afzelechin-pelargonidin 3-glucoside	7.86 ± 0.00	8.10 ± 0.03[a]	7.48 ± 0.22[b]	7.95 ± 0.37	3.55 ± 0.23[d]	3.41 ± 0.01[e]
3	Pelargonidin 3,5-diglucoside	0.40 ± 0.00	0.62 ± 0.00[a]	0.64 ± 0.02	0.64 ± 0.02	0.47 ± 0.02[d]	0.48 ± 0.02[e]
4	Epi-Afzelechin-pelargonidin 3-glucoside	2.14 ± 0.08	3.20 ± 0.08[a]	3.12 ± 0.12	3.19 ± 0.10	1.91 ± 0.05[d]	1.57 ± 0.01[e]
5	Cyanidin 3-galactoside	187.95 ± 0.09	61.48 ± 1.22[a]	56.83 ± 1.96[b]	45.23 ± 0.16[c]	29.74 ± 0.14[d]	22.62 ± 0.10[e]
6	Cyanidin 3-rutinoside	6.50 ± 0.07	3.61 ± 0.29[a]	3.10 ± 0.04[b]	3.17 ± 0.05	1.87 ± 0.08[d]	1.65 ± 0.15[e]
7	Pelargonidin 3-sambubioside	1.11 ± 0.00	1.50 ± 0.11[a]	1.03 ± 0.00[b]	0.95 ± 0.04[c]	0.15 ± 0.00[d]	0.15 ± 0.00[e]
8	Pelargonidin 3-glucoside	2113.59 ± 29.45	644.77 ± 4.24[a]	603.27 ± 7.74[b]	621.36 ± 2.97[c]	425.27 ± 16.61[d]	405.33 ± 1.25[e]
9	Pelargonidin 3-rutinoside	278.04 ± 0.95	62.13 ± 0.11[a]	72.55 ± 3.50[b]	70.14 ± 2.05[c]	51.35 ± 0.59[d]	46.60 ± 0.58[e]
10	Peonidin 3-glucoside	5.65 ± 0.04	1.10 ± 0.02[a]	1.07 ± 0.00	1.00 ± 0.02[c]	0.66 ± 0.02[d]	0.55 ± 0.03[e]
11	Pelargonidin dissacharide (hexose + pentose) acylated with acetic acid	1.35 ± 0.01	1.40 ± 0.08[a]	1.38 ± 0.07	1.40 ± 0.02	0.97 ± 0.05[d]	0.88 ± 0.07[e]
12	5-carboxypyranopelargonidin 3-glucoside	66.31 ± 0.53	16.03 ± 0.65[a]	19.69 ± 1.19[b]	19.12 ± 1.14	18.94 ± 0.67[d]	18.62 ± 0.99
13	Delphinidin 3-arabinoside	2.60 ± 0.15	5.24 ± 0.08[a]	4.31 ± 0.06[b]	4.03 ± 0.00[c]	3.99 ± 0.25[d]	3.85 ± 0.07[e]
14	Pelargonidin dissacharide (hexose + pentose) acylated with acetic acid	0.88 ± 0.00	1.39 ± 0.02[a]	1.12 ± 0.03[b]	0.95 ± 0.02[c]	0.76 ± 0.10[d]	0.68 ± 0.01[e]
15	Pelargonidin 3-arabinoside	0.84 ± 0.00	0.62 ± 0.06[a]	0.50 ± 0.00[b]	0.52 ± 0.01	0.24 ± 0.00[d]	0.09 ± 0.00[e]
16	Pelargonidin dissacharide (hexose + pentose) acylated with acetic acid	0.92 ± 0.00	0.61 ± 0.02[a]	0.59 ± 0.01	0.35 ± 0.00[c]	0.38 ± 0.00[d]	0.38 ± 0.01[e]
17	Pelargonidin 3-malonylglucoside	124.90 ± 0.58	21.45 ± 0.45[a]	21.45 ± 0.28	24.24 ± 0.19[c]	14.05 ± 0.84[d]	10.22 ± 0.94[e]
18	Cyanidin 3-(6-acetyl)-glucoside	1.83 ± 0.00	3.88 ± 0.05[a]	3.08 ± 0.19[b]	2.67 ± 0.07[c]	1.62 ± 0.01[d]	1.29 ± 0.13[e]
19	Delphinidin 3-galactoside	36.98 ± 0.01	20.94 ± 1.54[a]	17.86 ± 0.00[b]	17.56 ± 0.34[c]	16.07 ± 0.64[d]	16.05 ± 0.89[e]
20	Pelargonidin 3-(6-acetyl)-glucoside	149.75 ± 0.02	36.34 ± 0.13[a]	29.48 ± 0.01[b]	35.52 ± 0.08[c]	17.16 ± 0.30[d]	17.36 ± 0.46[e]
21	Cyanidin 3-glucoside	86.59 ± 0.01	41.02 ± 0.28[a]	35.66 ± 0.13[b]	34.85 ± 0.21[c]	35.51 ± 0.08[d]	31.74 ± 0.81[e]
22	Delphinidin 3-glucoside	1.05 ± 0.00	1.69 ± 0.07[a]	1.39 ± 0.00[b]	1.05 ± 0.01[c]	1.08 ± 0.08[d]	1.07 ± 0.09[e]
23	Pelargonidin 3-(6-succinyl)-arabinoside/ 3-(6-malonyl)-rhamnoside	32.86 ± 0.06	10.19 ± 0.10[a]	9.24 ± 0.03[b]	8.97 ± 0.17[c]	5.39 ± 0.11[d]	4.71 ± 0.02[e]

Different superscript letters mean significant differences ($p < 0.05$) between the samples as follows:
[a] Strawberry purée and 0;
[b] 0 and 15;
[c] 15 and 30;
[d] 30 and 60;
[e] 60 and 90.

Table 4 – pH, antioxidant activity and colour measurements of strawberry fermented beverage.

Sample	pH	ORAC (mmol Trolox/ mL beverage)	DPPH (IC50)	L*	a*	b*	C*	h	ΔE
0	2.52	107.810 ± 1.010	1.993 (1.783–2.228)*	77.814 ± 0.001	21.184 ± 0.055	27.148 ± 0.005	34.435 ± 0.037	52.035 ± 0.067	
R15	2.53	86.561 ± 3.169[a]	2.463 (2.154–2.817)	79.095 ± 0.001[a]	17.656 ± 0.009[a]	25.914 ± 0.005[a]	31.357 ± 0.001[a]	55.733 ± 0.019a	3.951
R30	2.53	101.004 ± 1.395[a]	2.531 (2.330–2.750)	78.957 ± 0.005[a]	14.744 ± 0.031[a]	26.603 ± 0.004[a]	30.415 ± 0.018[a]	61.005 ± 0.047a	6.563
R60	2.53	97.257 ± 2.039[a]	2.513 (2.350–2.687)	78.795 ± 0.067[a]	10.843 ± 0.035[a]	28.703 ± 0.129[a]	30.682 ± 0.133[a]	69.304 ± 0.234a	10.502
R90	2.53	96.596 ± 1.517[a]	3.638 (3.396–3.897)	79.668 ± 0.031[a]	9.068 ± 0.022[a]	29.015 ± 0.001[a]	30.399 ± 0.005[a]	72.645 ± 0.040a	12.398
F15	2.52	137.174 ± 1.980[a]	0.7443 (0.5190–1.067)	77.828 ± 0.025	20.895 ± 0.064[a]	27.159 ± 0.016	34.267 ± 0.052	52.427 ± 0.069a	0.289
F30	2.53	123.105 ± 0.163[a]	0.7951 (0.5004–163.2)	78.674 ± 0.002[a]	20.943 ± 0.010[a]	26.227 ± 0.020[a]	33.563 ± 0.022[a]	51.391 ± 0.008a	1.283
F60	2.52	102.763 ± 1.617[a]	1.1700 (1.007–1.359)	79.584 ± 0.006[a]	17.098 ± 0.056[a]	25.172 ± 0.045[a]	30.430 ± 0.068[a]	55.814 ± 0.039a	4.871
F90	2.54	101.904 ± 2.331[a]	1.467 (1.326–1.622)	79.049 ± 0.046[a]	16.616 ± 0.002[a]	25.860 ± 0.026[a]	30.738 ± 0.023[a]	57.278 ± 0.023a	4.904

Notes: Superscript letters mean significant differences ($p < 0.05$) with respect to initial samples.
Confidence interval expressed in brackets.
L^*: lightness.
a^* and b^* chromaticity coordinates indicate colour directions green ($-a^*$)/red ($+a^*$) and blue ($-b^*$)/yellow ($+b^*$).
$C^* = [a^{*2} + b^{*2}]^{1/2}$ indicates colour purity or saturation.
$H^* = \tan^{-1} b^*/a^*$ indicates sample colour.
ΔE: difference between two colours in an L*a*b* colour space.

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